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## Table of Contents

Cover.....	
SF 298.....	
Table of Contents.....	
Introduction.....	1
Body.....	1
Key Research Accomplishments.....	2
Reportable Outcomes.....	3
Conclusions.....	5
References.....	6
Appendices.....	6

## INTRODUCTION:

The **overall purpose** of the project is to use silkelastin-like polymers (SELPs) for the development of controlled gene delivery systems for localized breast cancer gene therapy. The **rationale** is that by controlling the structure of the polymer, it is possible to control DNA release, duration of transgene expression and the corresponding reduction in tumor size. Three Specific Aims were proposed:

- 1) To synthesize and characterize a series of SELP hydrogels
- 2) To examine the influence of polymer structure on DNA release *in vitro*.
- 3) To evaluate the influence of polymer structure on transfection efficiency and therapeutic efficacy *in vivo*.

Progress was made to partially accomplish Aims 1 and 3. In addition two related areas were explored as outlined in the body of this first year report.

## BODY:

### A) Biosynthesis of the proposed polymers:

For year 1 the following task and subtasks were proposed:

#### *Task 1. Synthesis of linear polymers*

- a. Design and synthesis of oligonucleotides encoding polymers
- b. Synthesis of monomer gene segments
- c. Synthesis of multimer gene segments
- d. Small scale expression and analysis of polymers
- e. Large scale fermentation of polymers
- f. Dissemination of data in a conference and preparation of report for year 1

We made progress in items (a), (b), and (c) and disseminated results in several publications and conferences (item f). This progress is summarized in Appendix 1 (See Scheme I and Figure 2 of this appendix and related methods and results). Item (d) is underway and item (e) is planned. Since stable cloning of multimers into expression vector (item d) proved to be difficult (no colonies observed) we have devised another strategy to accomplish this, i.e. a high molar ratio of monomers to vectors are being cloned to obtain colonies containing various multimer gene segments for subsequent expression. This work is under way.



### **B) In vivo evaluation of transfection efficiency:**

To ensure progress of research while the analogs were being biosynthesized, simultaneously we made partial progress in Aim 3 with a polymer analog that we had available in the lab (i.e., Polymer 47K). We carried out *in vivo* evaluation of transfection efficiency of a reporter gene with this polymeric analog in mice bearing breast tumor xenografts. This was originally planned for year 3.

Task proposed in Year 3 but accomplished in year 1 for *one polymer analog*:

*Task 1. In vivo* evaluation of transfection efficiency

- a. Induction of tumor growth and development
- b. Evaluation of transfection efficiency of reporter gene

The results of the *in vivo* study were published and are included in Appendix 2 (See Table 1 and Figures 6 and 7 and related methodologies, results and discussions.

In addition to progress in Aims 1 and Aim 3 described above, we also did additional experiments (not proposed in the original application-but related) which are summarized in sections C and D below.

### **C) Influence of DNA conformation, size and disk geometry on gene release:**

The purpose here was to evaluate, in addition to polymer structure proposed in the application, what other factors influence release of plasmid DNA. These results are also included in Appendix 2; please see Table 2, and Figures 2, 3, and 4 and the related methodologies, results and discussions.

### **D) Thermal characterization of SELP hydrogels:**

The aim here was to evaluate how is water structured within SELP hydrogels. Though this does not relate *directly* to the aims of this proposal, understanding water structure within SELP hydrogels can lead to understanding solute diffusion (including plasmid DNA) through the proposed hydrogels. The results of thermal characterization of SELP hydrogels are published and are included in Appendix 3.

### **KEY RESEARCH ACCOMPLISHMENTS:**

- a) Designed and synthesized oligonucleotides encoding the proposed polymers
- b) Synthesized monomer gene segments for SELP 415K
- c) Synthesized multimer gene segments for SELP 415K
- d) Cloned the multimer gene segments for small scale expression (under way)
- e) Evaluated *in vivo* the transfection efficiency of reporter gene released from one polymeric analog (SELP 47K)
- f) Evaluated the influence of DNA size and conformation on release
- g) Evaluated the influence of hydrogel geometry on DNA release
- h) Evaluated the structure of water in SELP 47K by thermal characterization

## **REPORTABLE OUTCOMES:**

### **A. Manuscripts:**

Total of two original research articles (Appendices 2 and 3), two invited review articles (Appendices 4 and 5) and two invited book chapters (Appendices 6 and 7) *pertaining to research described in this report.*

#### I) Original research articles:

**Appendix 2)** Z. Megeed, M. Haider, D. Li, B. W. O'Malley Jr., J. Cappello, and H. Ghandehari, In Vitro and In Vivo Evaluation of Recombinant Silk-Elastinlike Hydrogels for Cancer Gene Therapy, *Journal of Controlled Release*, 94, 433-445 (2004).

**Appendix 3)** Z. Megeed, J. Cappello, and H. Ghandehari, Thermal Analysis of Water in Silk-Elastinlike Hydrogels by Differential Scanning Calorimetry, *Biomacromolecules*, in press.

#### II) Invited review articles:

**Appendix 4)** M. Haider, Z. Megeed, and H. Ghandehari, Genetically Engineered Polymers: Status and Prospects for Controlled Release, *Journal of Controlled Release*, 95,1-26 (2004).

**Appendix 5)** M. Haider, and H. Ghandehari, Recombinant Polymers for Cancer Gene Therapy. *American Pharmaceutical Reviews*, submitted.

#### II) Invited book chapters:

**Appendix 6)** Z. Megeed, and H. Ghandehari, Genetically Engineered Protein-Based Polymers: Potential in Gene Delivery. *In Polymeric Gene Delivery: Principles and Applications*, M. Amiji (Ed.), CRC Press, Boca Raton, FL, in press.

**Appendix 7)** Z. Megeed, and H. Ghandehari, Recombinant Polymers for Drug Delivery. *In Polymeric Drug Delivery Systems*, G. Kwon (Ed.), Marcel Dekker, Inc. New York, NY, in press.

### **B) Presentations:**

#### I) Abstracts:

There were total of seven abstracts *pertaining to this research* during the reporting period. Of these three presentations were made by graduate students (one of which won a national award), and four by the PI (three of which were invited talks).

Z. Megeed, J. Cappello and H. Ghandehari, Thermal Characterization of Genetically Engineered Silk-Elastinlike Protein Polymer Hydrogels, Annual Meeting of the American Association of Pharmaceutical Scientists (AAPS), Salt Lake City, UT, October 26-30, 2003. (Student Poster Presentation)

H. Ghandehari, Z. Megeed, M. Haider, B.W. O'Malley Jr., D. Li, and J. Cappello, Gene Delivery from Recombinant Silk-Elastinlike Hydrogels, Symposium on Polymeric Drug Delivery: Science & Application, Fall American Chemical Society Meeting, New York, NY, September 7-11, 2003. (Invited Presentation by the PI)

M. Haider and H. Ghandehari, Size Dependent Release of Plasmid DNA from Silk-Elastinlike Hydrogels, Annual Meeting of the American Association of Pharmaceutical Scientists (AAPS), Salt Lake City, UT, October 26-30, 2003. (Student Poster Presentation)

**Winner of National AAPS Student Biotechnology Award:** Z. Megeed, J. Cappello, H. Ghandehari, Matrix-Mediated Controlled Delivery of Adenovirus and Plasmid DNA from Silk-Elastinlike Hydrogels, Annual Meeting of the American Association of Pharmaceutical Scientists (AAPS), Salt Lake City, UT, October 26-30, 2003. (Student Podium Presentation)

H. Ghandehari, Z. Megeed, M. Haider, and J. Cappello, Controlled Delivery of Bioactive Agents from Silk-Elastinlike Polymers, Third International Silk Conference, Montreal, Quebec, Canada, June 16 - 19, 2003. (Invited Presentation by the PI)

H. Ghandehari, Z. Megeed, M. Haider, D. Li, B. W. O'Malley Jr., and J. Cappello, Recombinant Polymers for Gene Delivery, Symposium on Recent Advances in Gene and Drug Delivery at the 2003 Regional Meeting of the American Chemical Society, Pittsburgh, PA, October 20, 2003. (Invited Presentation by the PI)

H. Ghandehari, Z. Megeed, M. Haider, R. Dandu, D. Li, B. W. O'Malley Jr., and J. Cappello. Localized Delivery of Plasmid DNA and Adenoviral Vectors For Cancer Gene Therapy By Recombinant Silk-Elastinlike Polymers, Sixth International Symposium on Polymer Therapeutics: From Laboratory to Clinical Practice, Welsh School of Pharmacy, Cardiff University, Cardiff, United Kingdom, January 7-9, 2004. (Podium/Poster Presentation by the PI- also an invited moderator in this conference)

## II) Invited Talks:

The PI gave the following invited presentations pertaining to this research in the reported period starting from the most recent:

University of Wisconsin, School of Pharmacy, Madison, Wisconsin, May 9, 2003.

Third International Conference on Silk, Montreal, Canada, June 16-19, 2003.

Symposium on Polymeric Drug Delivery: Science & Application at the Fall American Chemical Society Meeting, New York, NY, September 7-11, 2003.

Symposium on Recent Advances in Gene and Drug Delivery at the 2003 Regional Meeting of the American Chemical Society, Pittsburgh, PA, October 20, 2003.

Howard University School of Pharmacy, Washington DC, November 14, 2003.

**C) Degrees obtained that are supported by this award:**

*Zak Megeed* obtained his PhD in Fall of 2003. Currently he is a postdoctoral fellow at Harvard Center for Engineering and Medicine with a long-term goal of pursuing a courier in academia. He worked on the in vivo transfection studies and thermal characterization of water in SELP hydrogels. Zak won two awards as a result of his work in the past year: One was the National 2003 AAPS Graduate Student Biotechnology Award and the second a university – wide Dr. Arthur Schwartz Award for Academic Advancement based on academic excellence and interest in pursuing a career in science.

**CONCLUSIONS:**

In summary progress was made in the following areas:

**A)** In the biosynthesis of the analogs.

**B)** Demonstrated that when reporter plasmids embedded in one of the polymers are localized around breast cancer tumors in mice, show transfection efficiency over time depending on polymer concentration (4% showed better transfection over 21 days than 8% than 12%).

**C)** Release of plasmid DNA from the polymeric analogs depends on plasmid size (larger plasmids released to a lesser extent than smaller), DNA conformation (supercoiled and linear DNA released to a larger extent than circular) and hydrogel geometry (release was higher in disk gels than cylinder gels)

**D)** SELP-47K hydrogels contained non-freezable water. The heat capacity of water in these hydrogels is similar to that of "normal" water. Such results have implications in controlled release of plasmid DNA.

These results will allow us to: a) modify our cloning strategy to biosynthesize the polymers, b) easily conduct the in vivo studies with the new analogs. Overall this will aid in the tailor-making of the hydrogels for better localized transfection efficiency in gene therapy of breast cancer.

## REFERENCES:

None.

## APPENDICES:

**Appendix 1)** M. Haider, J. Cappello, and **H. Ghandehari**, Gene Delivery From Recombinant Silk-Elastinlike Hydrogels: In Vitro Release And Biosynthesis, 31<sup>st</sup> International Symposium on Controlled Release of Bioactive Materials, Honolulu, Hawaii, June 13-16, 2004. Proceedings.

**Appendix 2)** Z. Megeed, M. Haider, D. Li, B. W. O'Malley Jr., J. Cappello, and H. Ghandehari, In Vitro and In Vivo Evaluation of Recombinant Silk-Elastinlike Hydrogels for Cancer Gene Therapy, *Journal of Controlled Release*, 94, 433-445 (2004).

**Appendix 3)** Z. Megeed, J. Cappello, and H. Ghandehari, Thermal Analysis of Water in Silk-Elastinlike Hydrogels by Differential Scanning Calorimetry, *Biomacromolecules*, in press.

**Appendix 4)** M. Haider, Z. Megeed, and H. Ghandehari, Genetically Engineered Polymers: Status and Prospects for Controlled Release, *Journal of Controlled Release*, 95,1-26 (2004).

**Appendix 5)** M. Haider, and H. Ghandehari, Recombinant Polymers for Cancer Gene Therapy. *American Pharmaceutical Reviews*, submitted.

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**Appendix 7)** Z. Megeed, and H. Ghandehari, Recombinant Polymers for Drug Delivery. In *Polymeric Drug Delivery Systems*, G. Kwon (Ed.), Marcel Dekker, Inc. New York, NY, in press.

## GENE DELIVERY FROM RECOMBINANT SILK-ELASTINLIKE HYDROGELS: IN VITRO RELEASE AND BIOSYNTHESIS

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### ABSTRACT SUMMARY

The *in vitro* release of plasmid DNA as a function of size, conformation and disk geometry from silk-elastinlike (SELP) hydrogels was evaluated. The biosynthesis of SELP analogs by recombinant techniques for controlled gene delivery is described.

### INTRODUCTION

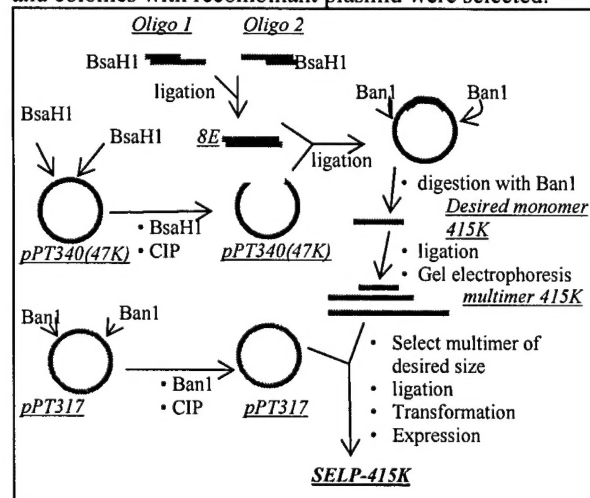
One way in which the safety and efficacy of gene delivery systems can be improved is through the use of polymeric matrices that control the rate and site of gene delivery. Genetic engineering techniques have enabled the synthesis of protein-based polymers with precisely controlled structures, well-defined molecular weights, monomer compositions, sequences, and stereochemistries.<sup>1</sup> Silk-elastinlike protein polymer (SELP) hydrogels are one class of genetically engineered polymers composed of repeating blocks of silk (GAGAGS) and elastin (GVGVP) units which confer flexibility, aqueous solubility and chemical and thermal stability to the polymer.<sup>2</sup> SELP copolymers with the appropriate sequence and composition undergo an irreversible sol-to-gel transition, which is accelerated at body temperature.<sup>3</sup> In this study we have investigated the effects of hydrogel geometry, DNA molecular weight, and DNA conformation on *in vitro* DNA release from SELP-47K hydrogel<sup>4</sup> (amino acid sequence in figure 1a, molecular weight (mw)  $\approx$  69.82 kda). In addition, in order to evaluate the influence of polymer structure on release, we report the progress in the biosynthesis of SELP-415K (expected amino acid sequence in figure 2a) containing a longer elastin block per repeating unit.

### EXPERIMENTAL METHODS

**Preparation of SELP-47K hydrogels containing plasmid DNA and *in vitro* release studies.** SELP-47K hydrogels containing DNA were prepared by mixing polymer and plasmid DNA in phosphate buffer saline (PBS) to final concentration of 250  $\mu$ g/ml DNA in 10 wt% polymer. After 4 hours of cure time at 37 °C the hydrogels were cut into cylindrical discs and set in glass vials containing PBS (pH 7.4, 0.01% NaN<sub>3</sub>, 0.17 M NaCl) at 37 °C in a shaking (120 rpm) incubator for 28 days. Sampling and replacement of the release buffer was carried out at predetermined time points. The concentration of DNA in the release medium was determined using the PicoGreen reagent. The effect of plasmid molecular weight on release from SELP hydrogels *in vitro* was studied using plasmids DNA of different sizes (Table 1). To study the influence of DNA conformation on release, open-circular and linearized pRL-CMV-luc were obtained by digestion with *N.Bst*NIa and *Hind* III enzymes respectively. The average effective diffusivities of plasmids within the

hydrogels were determined using a mathematical model describing diffusion from a cylinder in both the radial and axial directions.<sup>5,6</sup>

**Genetic engineering of SELP-415K analogs.** Oligonucleotides encoding for 8 elastin units (8E) were obtained from IDTdna (Coralville, IO). The synthesis of SELP-415K polymers followed a random concatemerization procedure (Scheme 1) starting with cloning vector pPT340 carrying sequence encoding SELP-47K (Protein Polymer Technology Inc, San Diego, CA). After ligation of oligonucleotide 8E to the cloning vector pPT340, vectors were transformed to *DH-5 $\alpha$*  chemically competent *E. coli* (Promega, Madison, WI) and colonies with recombinant plasmid were selected.



**Scheme I.** Genetic engineering of SELP-415 (CIP calf intestinal phosphatase).

The plasmids were then isolated, purified and digested with *Ban*I restriction enzyme yielding the desired monomer gene segment 415K. Monomers 415K were then self-ligated and the ligation mixture was eluted on agarose gel for size fractionation of the resulting multimers which are to be ligated to pPT317 vector (Protein Polymer Technology Inc, San Diego, CA) for expression.

### RESULTS AND DISCUSSION

DNA release data indicate that the relative rate of release of each plasmid was inversely related to its molecular weight (Table 1). The size-dependent release of plasmid DNA can be explained by the hindered diffusion of larger plasmids through the pores that are formed by physical crosslinking of SELP-47K.

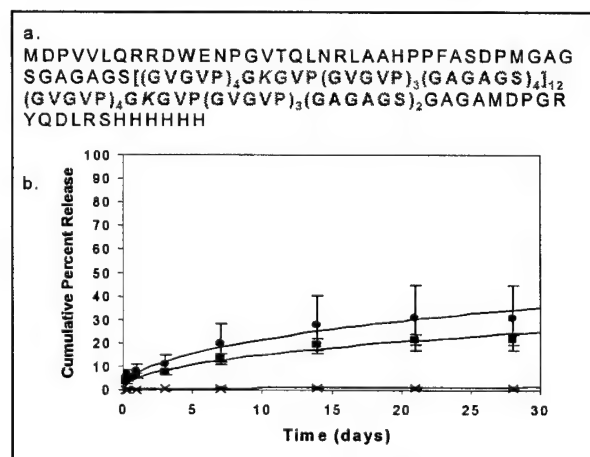
While many studies have indicated a preference for supercoiled DNA for greater transfection efficiency, at least one reported that the delivery of linear DNA results

in prolonged *in vivo* transgene expression.<sup>7</sup> The percentage cumulative release of the different conformations of pRL-CMV over 28 days was on the order of linear > supercoiled > open circular (Figure 1b).

**Table 1.** Characteristics of plasmid DNA-containing SELP hydrogels for release experiments

Plasmid	Geometry	Size (kbp) <sup>a</sup>	HSA <sup>b</sup> (cm <sup>2</sup> )	De <sup>c</sup> (cm <sup>2</sup> /sec)
pUC18	Disc	2.60	0.74	$2.55 \pm 0.51 \times 10^{-10}$
<i>l</i> -pRL-CMV <sup>d</sup>	Disc	4.08	0.74	$1.94 \pm 0.27 \times 10^{-10}$
<i>sc</i> -pRL-CMV <sup>e</sup>	Disc	4.08	0.74	$8.90 \pm 0.12 \times 10^{-11}$
<i>oc</i> -pRL-CMV <sup>f</sup>	Disc	4.08	0.74	$1.96 \pm 0.83 \times 10^{-13}$
pRL-CMV	Large Disc	4.08	1.70	$1.76 \pm 0.28 \times 10^{-10}$
pRL-CMV	Cylinder	4.08	1.07	$9.23 \pm 0.15 \times 10^{-11}$
pCFB-EGSH-Luc	Disc	8.50	0.74	$3.09 \pm 0.43 \times 10^{-11}$
pFB-ERV	Disc	11.00	0.74	$1.70 \pm 0.52 \times 10^{-12}$

<sup>a</sup> kbp = kilobase pairs, <sup>b</sup> HSA = hydrogel surface area, <sup>c</sup> De = average effective diffusivity of plasmid DNA in hydrogel, <sup>d</sup> l = linearized, <sup>e</sup> sc = supercoiled, <sup>f</sup> oc = open-circular.



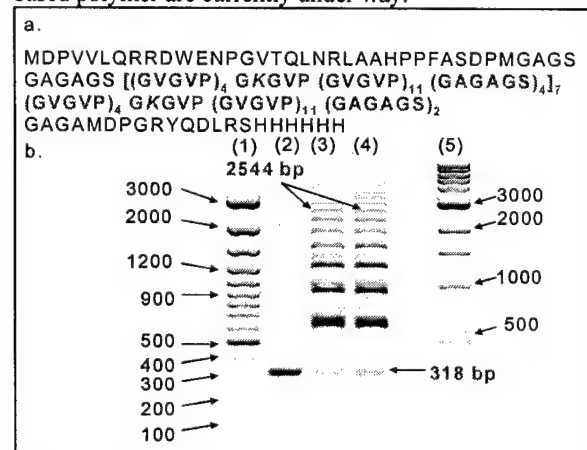
**Figure 1.** a) Amino acid sequence of SELP- 47K; b) Effect of pRL-CMV conformation on in vitro release from SELP-47K hydrogels: (●) Linear, (■) Supercoiled, (×) Open-circular; (—) Theoretical release based on diffusion Equation.(n=3).

Diffusion of linearized plasmid DNA from polymeric matrices involves both free diffusion and reptation mechanisms, which would be expected to increase its rate of release from SELP-47K hydrogels, as the pore size would be less restrictive to a semi-flexible, linearized molecule. The lower rate of release of supercoiled DNA from SELP-47K hydrogel can be attributed to its larger diameter which likely increase the contact between this molecule and the hydrogel network. The essential lack of release of the open-circular form of pRL-CMV may be due to its impalement on the fibers forming the hydrogel matrix some of the physically cross-linked polymers

extend through the open-circular plasmid preventing transport of the DNA.

The study of the influence of hydrogel geometry on plasmid release showed that the cumulative release from the large disc geometry was greater than that released from the cylindrical geometry (Table 1). The difference in the rate of release can be attributed to the larger surface-to-volume ratio of the disk-like hydrogels.

In order to study the effect of polymer structure on the release of plasmid DNA from SELP hydrogels, we are in the process of synthesizing block copolymers containing larger elastin blocks by recombinant techniques. DNA sequencing has confirmed the successful cloning of monomer gene segment of 415K consisting of 318 bp. Self-ligation of monomer gene segments resulted in high molecular weight products larger than 318 bp (Figure 2b). Multimers of desired size (2544 bp) encoding for a SELP-415K (mw ≈ 71.5 kda) were recovered as bands corresponding to this molecular weight. Further ligation into the expression vector and expression of the protein-based polymer are currently under way.



**Figure 2.** a) Amino acid sequence of SELP-415K; b) Agarose gel electrophoresis of monomer self-ligation, lane (1) 100bp ladder, (2) SELP-415K monomer gene segment, (3,4) self-ligation mixture and (5) 1kb DNA ladder.

## CONCLUSION

These studies have demonstrated that the release of plasmid DNA from recombinant silk-elastinlike hydrogels is size- and conformation-dependent. The synthesis of SELP415K will further illustrate the effect of polymer structure on localized gene release and delivery.

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GENE DELIVERY

## In vitro and in vivo evaluation of recombinant silk-elastinlike hydrogels for cancer gene therapy

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### Abstract

The objectives of this study were to evaluate: (i) the influences of hydrogel geometry, DNA molecular weight, and DNA conformation on DNA release from a silk-elastinlike protein polymer (SELP) hydrogel, (ii) the bioactivity and transfection efficiency of encapsulated DNA over time in vitro, (iii) the delivery and transfection of a reporter gene in a murine model of human breast cancer in vivo, and (iv) the in vitro release and bioactivity of adenovirus containing the green fluorescent protein (gfp) gene as a marker of gene transfer. Plasmid DNA was released from SELP hydrogels in a size-dependent manner, with the average effective diffusivity ranging from  $1.70 \pm 0.52 \times 10^{-12} \text{ cm}^2/\text{s}$  for a larger plasmid (11 kbp) to  $2.55 \pm 0.51 \times 10^{-10} \text{ cm}^2/\text{s}$  for a smaller plasmid (2.6 kbp). Plasmid conformation also influenced the rate of release, with the rank order linear > supercoiled > open-circular. DNA retained bioactivity in vitro, after encapsulation in a SELP hydrogel for up to 28 days. Delivery of pRL-CMV from a SELP hydrogel resulted in increased transfection in a murine model of human breast cancer by 1–3 orders of magnitude, as compared to naked DNA. The release of a bioactive adenoviral vector was related to the concentration of the polymer in the hydrogel. These studies indicate that genetically engineered SELP hydrogels have potential as matrices for controlled nonviral and viral gene delivery.

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**Keywords:** Genetically engineered polymers; Silk-elastinlike protein polymers; Controlled gene delivery; Cancer gene therapy; Adenoviral gene delivery

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### 1. Introduction

One of the major obstacles for successful cancer gene therapy is the lack of safe and clinically effective gene delivery systems [1]. One way in which the safety and efficacy of gene delivery systems can be improved is through the use of polymeric matrices that control the rate and location of gene delivery [2–4]. Although naked DNA has



primarily been delivered from these systems, recent efforts have explored the localized matrix-mediated delivery of viral vectors as well [5–7]. Generally biomaterials used in controlled gene delivery are composed of polymers synthesized by chemical methods or obtained from natural sources. Chemical synthesis generally produces random copolymers with unspecified monomer sequences and statistical distributions of molecular weights [8]. This heterogeneity can yield inconsistency in the biological fate and mechanical properties of such polymers. Moreover, chemically synthesized polymers may contain residual organic solvents, which adversely affect DNA and/or viral vectors. Naturally-derived collagen matrices, which have been used in adenoviral delivery, are limited by batch to batch variability, limited control over crosslinking density and therefore release, and difficulty in introducing new or modified functions (e.g. stimuli-sensitivity, biodegradation, and biorecognition) [6,7].

Advances in recombinant DNA technology has allowed the biological synthesis of engineered protein polymers containing repeating blocks of amino acids with precise composition, sequence, and length [9–11]. Biologically synthesized polymers do not contain toxic monomer residues and solvents and, depending on their structure, can biodegrade to nontoxic amino acids and be eliminated at controlled rates from the body. Silk-elastinlike polymers (SELPs) comprise a class of genetically engineered biomaterials composed of amino acid motifs from two naturally occurring proteins: *Bombyx mori* (silkworm) silk (GAGAGS) and mammalian elastin (GVGVVP) [9]. By combining the silk-like and elastin-like blocks in various ratios and sequences, it is possible to produce an assortment of biomaterials with diverse material properties. The biological synthesis and characterization of biodegradable and stimuli-sensitive SELP copolymers for controlled drug delivery applications has been a subject of research in our laboratories [12–17]. SELP copolymers with the appropriate sequence and composition undergo an irreversible sol-to-gel transition, which is accelerated at body temperature [12,15]. The aqueous polymeric solutions are thus liquid at room temperature and form a firm yet pliable hydrogel in situ minutes after injection. The ability to localize the hydrogel by injection through a needle or endoscope avoids more invasive surgical implantation procedures

necessary for other pre-formed, solid polymeric matrices. Once a gel has formed, drug or gene delivery agents can be released while the polymer matrix degrades.

The ability to precisely introduce functional motifs into SELP copolymers using genetic engineering techniques provides control over gelation, crosslinking density, biodegradation, biorecognition, and stimuli-sensitivity [18]. The sol-to-gel transition of these copolymers allows DNA or viral particles to be loaded into an aqueous solution at room temperature, and the solution to be injected by minimally invasive procedures to form solid implants in and around the tumor at body temperature. Furthermore, previous studies have shown that SELP hydrogels are biocompatible and induce a relatively low immunogenic response [19]. Systems with these properties would provide controlled delivery of plasmid DNA and viral particles for localized cancer gene therapy applications.

Previously, we reported the release of plasmid DNA from SELP matrices in aqueous buffer [17]. Here we report the characterization of the in vitro and in vivo delivery of naked DNA and an adenoviral vector. These studies can lead to the development of novel therapeutic gene delivery systems and to the design of new recombinant biomaterials tailor-made for specific gene delivery needs.

## 2. Materials and methods

### 2.1. Materials

The silk-elastinlike protein copolymer SELP-47K, composed of four silk-like blocks, seven elastin-like blocks, and one modified elastin block containing a lysine (K) substitution (amino acid sequence in Fig. 1), was obtained from Protein Polymer Technologies (San Diego, CA) as 12 wt.% solutions in 3 ml syringes and stored at –80 °C until use. Plasmid pRL-CMV (4.08 kbp), the *Renilla* luciferase assay, and *Escherichia coli* JM109 were purchased from Promega (Madison, WI). Plasmids pUC18 (2.6 kbp), pCFB-EGSH-Luc (8.5 kbp), pFB-ERV (11 kbp), and *E. coli* XL 10 Gold were purchased from Stratagene (Cedar Creek, TX). PicoGreen DNA quantitation reagent

MDPVVLQRDWDENPGVTQLNRLAAHPFASDPMGAGSGAGAGS  
**[(GVGVVP)<sub>4</sub>GKGVVP(GVGVVP)<sub>3</sub>(GAGAGS)<sub>4</sub>]<sub>12</sub>**  
 (GVGVVP)<sub>4</sub>GKGVVP(GVGVVP)<sub>3</sub>(GAGAGS)<sub>2</sub>GAGAMDPGRYQDLRSHHHHHH

Fig. 1. The amino acid sequence of SELP-47K. The 884 amino acids have a molecular weight of 69,814 Da. It is composed of head and tail portions, and a series of silk-like (GAGAGS) and elastin-like (GVGVVP) repeats (primary repetitive sequence in bold). Abbreviation key: A, alanine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; K, lysine; L, leucine; M, methionine; N, asparagine; R, arginine; P, proline; Q, glutamine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

was purchased from Molecular Probes (Eugene, OR). *N.Bst*NI nicking enzyme and *Hind* III restriction endonuclease were purchased from New England Biolabs (Beverly, MA). Qiafilter Giga and Endo-free Giga kits were purchased from Qiagen (Valencia, CA). Dulbecco's phosphate buffered saline (PBS), Dulbecco's Modified Eagles Medium (DMEM), Hank's Balanced Salt Solution (HBSS), fetal bovine serum (FBS), trypsin–EDTA, and penicillin–streptomycin (10,000 U/ml penicillin, 10,000 µg/ml streptomycin) were purchased from Invitrogen (Carlsbad, CA). COS-7 and HEK-293 cells were purchased from the American Type Culture Collection (Manassas, VA). The Non-Interfering Protein Assay was purchased from Geno Technologies (St. Louis, MO). Athymic, female, *nu/nu* mice, 5–6 weeks in age, were purchased from Harlan Bioproducts (Indianapolis, IN). The human breast cancer cell line MDA-MB-435 was a gift from Dr. A. James Mixson, of the University of Maryland School of Medicine. Syringes and needles, manufactured by BD Medical Supplies (Franklin Lakes, NJ), were purchased from VWR (West Chester, PA). Avertin stock solution was prepared by mixing 2,2,2-tribromoethanol (Sigma-Aldrich, St. Louis, MO) and *tert*-amyl alcohol (Sigma-Aldrich) at a weight to volume ratio of 1:1 (g/ml). For use in animals, the stock Avertin solution was diluted to 2.5 vol%, in sterile, endotoxin-free water (Biosource International, Camarillo, CA). Lysing Matrix D was purchased from QBiogene (Carlsbad, CA). Adenoviral vector pAd1.CMV was a gift of Savio Woo, Mount Sinai Hospital, New York, NY. The pJM17 helper plasmid was purchased from Microbix (Toronto, Ontario, Canada). Statistical analyses were performed in SPSS v10.0 (SPSS, Chicago, IL). *Mathematica* software, used for nonlinear regression, was

purchased from Wolfram Research (Champaign, IL). All other reagents were purchased from Sigma-Aldrich.

## 2.2. Plasmid DNA preparation and purification

Plasmid pRL-CMV was propagated in *E. coli* JM109, while pUC18, pCFB-EGSH-Luc and pFB-ERV were propagated in *E. coli* XL 10 Gold. For in vitro studies, plasmid DNA was purified using a Qiagen Giga Kit according to manufacturer's instructions. The concentration and purity of the plasmids was verified by UV spectrophotometry (Ultrospec 4000, Amersham Biosciences, Piscataway, NJ) at 260 and 280 nm. The ratio of  $A_{260}/A_{280}$  was in the range of 1.8–2.0 for all plasmids. Plasmids were electrophoresed on a 0.9% agarose gel and stained with ethidium bromide to verify the absence of genomic DNA and the integrity of the plasmid. For in vivo studies, an Endo-free Giga Kit was used for plasmid preparation.

## 2.3. Generation of linear and open circular pRL-CMV

Supercoiled pRL-CMV was linearized by cleavage at base pair 754 of the plasmid with *Hind* III restriction endonuclease. Two-hundred units of *Hind* III were used to cleave 200 µg of pRL-CMV at 37 °C for 1 h. The linearized DNA was purified by phenol-chloroform extraction, concentrated by ethanol precipitation, and analyzed by agarose gel electrophoresis.

Open circular pRL-CMV was prepared using *N.Bst*NI nicking enzyme, which hydrolyzes only one strand of the plasmid to produce plasmids that are “nicked”, rather than cleaved. This results in plasmids that are in an open-circular conformation. The plasmid DNA (200 µg) was treated with *N.Bst*NI at 55 °C for 1 h, purified by phenol-chloroform extraction, concentrated by ethanol precipitation, and analyzed by agarose gel electrophoresis.

## 2.4. Preparation of SELP-47K hydrogels containing plasmid DNA for in vitro release studies

Syringes containing frozen 12 wt.% SELP-47K solution were thawed in a beaker containing 500 ml

of water for 5 min at room temperature. Mixtures of polymer and DNA were produced by gently mixing the polymer with the plasmid DNA of the appropriate concentration. The volume of the mixture was adjusted by addition of PBS and MilliQ water to yield 250 µg/ml plasmid DNA in 10 wt.% polymer. The polymer/DNA solution was then transferred to disposable syringes (1 or 3 ml), incubated at 37 °C for 4 h, and allowed to gel. After 4 h, the hydrogels in the syringes were cut into cylindrical discs using a razor blade.

### 2.5. Release of plasmid DNA from the hydrogels

Hydrogels were placed in 4 ml glass vials containing 3 ml of 10 mM phosphate buffer (pH 7.4, 0.01% NaN<sub>3</sub>, 0.17 M NaCl). Vials were incubated at 37 °C in a shaking (120 rpm) incubator for 28 days. Sampling and replacement of the release buffer was carried out at predetermined time points. The concentration of DNA in the release medium was determined using the PicoGreen reagent. A standard curve of plasmid concentration was obtained for each assay. Results are mean ± standard deviation (S.D.) ( $n=3$ ).

### 2.6. Calculation of apparent diffusivity of plasmids in the hydrogels

Each set of release data were charted in terms of the cumulative fraction released versus time. The average effective diffusivity of the plasmid within the hydrogels was determined using the following equation, which describes diffusion from a cylinder in both the radial and axial directions [20,21]:

$$\frac{M_t}{M_\infty} = 1 - \frac{32}{\pi^2} \sum_{i=1}^{\infty} \frac{1}{\alpha_i^2} \exp\left(-\frac{\alpha_i^2}{r^2} D_e t\right) \times \sum_{j=0}^{\infty} \frac{1}{(2j+1)^2} \exp\left(-\frac{(2j+1)^2 \pi^2}{h^2} D_e t\right) \quad (1)$$

In this equation,  $r$  is the radius of the cylinder,  $h$  is the height of the cylinder,  $M_t$  is the cumulative amount of solute released at time  $t$ ,

$M_\infty$  is the amount released as  $t \rightarrow \infty$ ,  $D_e$  is the average effective intra-gel diffusivity of the solute, and  $\alpha_i$  are the roots of the zero-order Bessel function,  $J_0(\alpha_i)=0$ .  $D_e$  was estimated from a nonlinear fit of Eq. (1) to the experimental release data, using *Mathematica* software. The  $R^2$  values for each fit were the ratio of the difference between the corrected total sum of squares and the residual sum of squares to the corrected total sum of squares. Eq. (1) assumes that the diffusion coefficient is independent of solute concentration, that the concentration of solute at the surface of the hydrogel is effectively zero, that no convection occurs, and that there are no polymer–solute interactions. Under the conditions studied here, these assumptions are valid for SELP-47K hydrogels containing plasmid DNA [17]. Results are mean ± S.D. ( $n=3$ ).

### 2.7. In vitro bioactivity of plasmid DNA after encapsulation in SELP-47K hydrogels

Hydrogels containing pRL-CMV were prepared by previously described methods and incubated in PBS at 37 °C for 28 days [17]. At weekly time points, DNA was extracted from the hydrogels by phenol–chloroform extraction. After concentration by ethanol precipitation, DNA was analyzed by electrophoresis on a 1% agarose gel.

Bioactivity of the extracted DNA was evaluated by in vitro transfection assays performed on COS-7 (African Green Monkey) cells. The day prior to transfection, COS-7 cells were plated in 12-well plates containing 1 ml of DMEM supplemented with 10 vol% FBS at a density of  $2 \times 10^5$  cells per well. On the day of transfection, 1 µg of DNA extracted from the hydrogels was used to transfect the COS-7 cells of a single well by mixing with 2.5 µl Lipofectamine 2000 per manufacturer's instructions. Forty-eight hours after transfection, bioactivity of the plasmid DNA was evaluated by the *Renilla* Luciferase Assay, using a Wallac Victor 1420 microplate reader (PerkinElmer, Wellesley, MA). Luminescence results were normalized for the number of cells by measuring the total protein (TP) content of each well with the Non-Interfering Protein Assay. Results are mean ± S.D. ( $n=3$ ).

## 2.8. In vivo delivery of the *Renilla luciferase* gene from SELP-47K hydrogels

Animal studies were conducted with female athymic *nu/nu* mice 5–6 weeks in age, under the approval and guidelines of the University of Maryland School of Medicine Institutional Care and Animal Use Committee (protocol #0402009). MDA-MB-435 cells were cultured in 150 cm<sup>2</sup> flasks containing DMEM supplemented with 10 vol% FBS and 1 vol% penicillin–streptomycin to approximately 85–90% confluence. Cells were harvested with trypsin–EDTA and counted on a hemacytometer while being washed twice with HBSS to remove traces of FBS and trypsin. Cells were suspended in HBSS, to a final concentration of approximately  $1 \times 10^7$  cells/ml. To induce tumors, 100  $\mu$ l ( $1 \times 10^6$  cells) of the cell suspension was injected subcutaneously in the back of each mouse just inferior to the shoulder. Tumors of appropriate size for intratumoral injection ( $\sim$  8 mm diameter) were obtained after approximately 14–21 days.

Prior to intratumoral injection, animals were anesthetized by intraperitoneal injection of 0.02 ml of 2.5 vol% Avertin per gram of body weight. Animals received a single intratumoral injection containing 100  $\mu$ l of either polymer solution with DNA, polymer solution alone, or polymer vehicle (0.71  $\times$  PBS) with DNA (referred to as *naked DNA*). All treatment groups receiving DNA (Table 1, Groups 2–5) contained five animals per time point. The control group, receiving polymer only (Table 1, Group 1), contained three animals per time point. DNA was administered at a single dose of 70  $\mu$ g pRL-CMV in 100  $\mu$ l injection volume. The dose and volume of injections were chosen to be optimal for intratumoral injection of naked DNA [22].

Intratumoral injections were performed manually by elevating the skin 1 cm posterior to the tumor and inserting a 25 gauge needle through the skin. The needle tip was advanced to the center of the tumor and 100  $\mu$ l of the appropriate solution was slowly injected. After injection, the needle was withdrawn quickly and slight pressure was applied to the skin for 5–10 s. This procedure resulted in intratumoral injections with no observed backflow to the surface of the skin.

At the predetermined time points (Table 1), animals were euthanized by carbon dioxide asphyxiation and tumors were resected. In addition to resecting the tumor, the skin surrounding the tumor ( $\sim$  1 cm radius) was separately resected. Tumor and skin were placed in separate Lysing Matrix D tubes and stored at  $-80^\circ\text{C}$  until analysis.

Tumor and skin specimens were defrosted at room temperature and 800  $\mu$ l of  $2 \times$  lysis buffer from the *Renilla Luciferase Assay Kit* was added to each tissue sample. Tissues were homogenized twice using a Fast Prep FP120 tissue homogenizer (QBiogene) at setting 4.5 for 40 s each. Tissues were cooled on ice for 5 min between homogenization cycles. After homogenization, samples were centrifuged to remove insoluble debris for 1 min at  $14,000 \times g$ .

Luciferase expression was measured on a Mono-light 2010 luminometer (Analytical Luminescence Laboratories, San Diego, CA) by adding 100  $\mu$ l of luciferase assay reagent to 25  $\mu$ l of tumor or skin homogenate. The background luminescence of tumors receiving 8 wt.% polymer with no DNA was subtracted from the luminescence of tumors receiving polymer with DNA. The instrument background luminescence was subtracted from luminescence of tumors receiving naked DNA. Luminescence measurements were subsequently normalized for TP content of the tumor and skin homogenates determined by the Non-Interfering Protein Assay. Transfection data are expressed as relative light units (RLU) per milligram of TP.

The Kolmogorov–Smirnov statistical test for normality was performed on data from each group and time point [23]. Outliers were identified by constructing box and whisker plots. Values greater than 1.5 times outside the whiskers were considered outliers and were eliminated from the data set prior to tests for statistical significance [24]. A maximum of one data point was

Table 1  
Treatment groups, time points, and number of animals for in vivo experiments

Group	Treatment	Time points (days)	Number of animals
1	8 wt.% polymer only	3, 7, 14, 21, 28	15
2	4 wt.% polymer + DNA	3, 7, 14, 21, 28	25
3	8 wt.% polymer + DNA	3, 7, 14, 21, 28	25
4	12 wt.% polymer + DNA	3, 7, 14, 21, 28	25
5	Naked DNA	3, 7, 14, 21, 28	25

removed per group and time point resulting in a minimum number of replicates of four ( $n=4$ ) for each data set. The Mann–Whitney test was used to compare results between groups, time points, and tissues [23]. Statistical significance was defined as  $P<0.05$ .

### 2.9. Adenovirus release and bioactivity

An adenovirus containing the green fluorescent protein gene (AdGFP) was engineered by cloning the GFP gene into the pAd1.CMV plasmid followed by co-transfection into HEK-293 cells with the helper plasmid pJM17 [25]. The virus was mixed with 12 wt.% polymer solutions and polymer diluent in amounts appropriate to obtain 4, 8, and 11.3 wt.% polymer solutions containing  $1.2 \times 10^7$  pfu/ml. Virus-containing polymer solutions were placed in 1 ml syringes and cured for 4 h at 37 °C. After curing, 50  $\mu$ l volumes were cut from the hydrogel using a sterile razor blade. Each hydrogel contained approximately  $6 \times 10^5$  pfu.

Hydrogel discs were placed in 1 ml of PBS release medium, incubated at 37 °C, and mildly agitated at 120 rpm for 28 days. In addition, vials containing  $6 \times 10^5$  pfu in 1 ml of release medium were incubated as controls. Release and bioactivity were tested by cell-based assays. Twenty-four hours prior to the assay, HEK-293 cells were plated in 96-well plates at a density of  $3 \times 10^3$  cells per well in 200  $\mu$ l DMEM with 10 vol% FBS and 1 vol% penicillin–streptomycin. At specified time points,

50  $\mu$ l of release medium was removed from the 4, 8, and 11.3 wt.% polymer hydrogels and controls. The culture medium was removed from the HEK-293 cells and the 50  $\mu$ l aliquot of release medium was placed on the cells and incubated at 37 °C for 45 min. Subsequently, 150  $\mu$ l of culture medium was added to each well. The cells were incubated at 37 °C for 48 h and infected cells were observed by fluorescence microscopy.

## 3. Results and discussion

### 3.1. Effect of plasmid size on in vitro release

The influence of plasmid molecular weight on release from SELP-47K hydrogels was determined using hydrogels containing plasmids of the following sizes: pUC18 (2.6 kbp), pRL-CMV (4.08 kbp), pCFB-EGSH-Luc (8.5 kbp), and pFB-ERV (11 kbp) (Table 2). After a 4 h cure time, hydrogel discs containing 10 wt.% polymer and 250  $\mu$ g/ml of plasmid DNA were prepared. The properties of the hydrogels and the calculated average effective diffusivities of the respective plasmids are reported in Table 2. The hydrogel discs had a radius of 2.3 mm and a height of 2.8 mm; cylinders were 2.3 mm in radius and 5.1 mm in height; large discs were 4.3 mm in radius and 2.0 mm in height.

The relative rate of release of each plasmid was inversely related to its molecular weight. The smallest

Table 2  
Characteristics of plasmid DNA-containing silk-elastinlike hydrogels for release experiments

Plasmid	Geometry	Plasmid size (kbp) <sup>a</sup>	Hydrogel surface area (cm <sup>2</sup> )	$D_e^b$ (cm <sup>2</sup> /s)
pUC18	Disc	2.60	0.74	$2.55 \pm 0.51 \times 10^{-10}$
<i>l</i> -pRL-CMV <sup>c</sup>	Disc	4.08	0.74	$1.94 \pm 0.27 \times 10^{-10}$
<i>sc</i> -pRL-CMV <sup>d</sup>	Disc	4.08	0.74	$8.90 \pm 0.12 \times 10^{-11}$
<i>oc</i> -pRL-CMV <sup>e</sup>	Disc	4.08	0.74	$1.96 \pm 0.83 \times 10^{-13}$
pRL-CMV	Large disc	4.08	1.70	$1.76 \pm 0.28 \times 10^{-10}$
pRL-CMV	Cylinder	4.08	1.07	$9.23 \pm 0.15 \times 10^{-11}$
pCFB-EGSH-Luc	Disc	8.50	0.74	$3.09 \pm 0.43 \times 10^{-11}$
pFB-ERV	Disc	11.00	0.74	$1.70 \pm 0.52 \times 10^{-12}$

Samples in which the conformation is not denoted were a mixture of the three—primarily supercoiled—as isolated from the Gigaprep column.

<sup>a</sup> kbp, kilobase pairs.

<sup>b</sup>  $D_e$ , average effective diffusivity of plasmid DNA in hydrogel determined by nonlinear fit of release data to Eq. (1).

<sup>c</sup> *l*, linearized.

<sup>d</sup> *sc*, supercoiled.

<sup>e</sup> *oc*, open-circular.

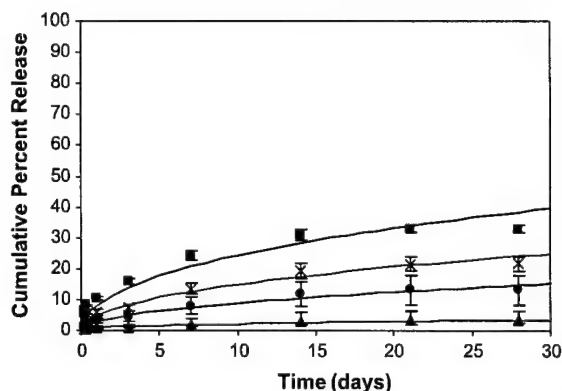


Fig. 2. Effect of plasmid DNA size on in vitro release from SELP-47K hydrogels: (■) pUC 18 (2.6 kbp), (×) pRL-CMV (4.08 kbp), (●) pCFB-EGSH-Luc (8.5 kbp), (▲) pFB-ERV (11 kbp); (—) theoretical release based on Eq. (1). Each data point represents the mean  $\pm$  S.D. for  $n=3$  samples.

plasmid, pUC18 was released fastest, followed by pRL-CMV, pCFB-EGSH-Luc, and the largest plasmid pFB-ERV, which showed less than 5% release over 28 days (Fig. 2). To calculate the average effective diffusivities of the plasmids within the SELP-47K hydrogel, the experimental release data were fit to Eq. (1). The average effective diffusivity of plasmid pUC18 in 10 wt.% SELP-47K hydrogels was  $2.55 \pm 0.51 \times 10^{-10}$  cm<sup>2</sup>/s and the  $R^2$  value was 0.93. Plasmids pRL-CMV and pCFB-EGSH-Luc showed lesser effective diffusivities of  $8.90 \pm 0.12 \times 10^{-11}$  and  $3.09 \pm 0.43 \times 10^{-11}$  cm<sup>2</sup>/s, respectively. For both plasmids, the  $R^2$  values obtained after fitting to Eq. (1) were 0.97. For the largest plasmid, pFB-ERV, the calculated diffusivity was  $1.70 \pm 0.52 \times 10^{-12}$  cm<sup>2</sup>/s and the  $R^2$  value was 0.86.

The size-dependent release of plasmid DNA can be explained by the hindered diffusion of larger plasmids through the pores that are formed by physical cross-linking of SELP-47K. These results are consistent with a previous study in which size-dependent release of hydrophilic bioactive agents from SELP-47K was observed [16].

### 3.2. Effect of plasmid conformation on release

The conformation of plasmid DNA, namely supercoiled, open-circular, or linear has been hypothesized to play a role in the transfection efficiency of the plasmids. While conventional thinking has generally

indicated a preference for supercoiled DNA, at least one study has shown that the delivery of linear DNA results in prolonged transgene expression in vivo [26]. Other work has shown that the conformation of DNA is largely irrelevant to the transfection efficiency in vitro and in vivo [27]. It is possible that the conformational requirements of DNA delivery may depend on the delivery system, whether the delivery is taking place in vitro or in vivo, and the concentration of nucleases at the site of delivery.

In order to evaluate the influence of conformation on DNA release from SELP-47K hydrogels, plasmid DNA predominantly in the supercoiled, open-circular, and linear conformations were produced and their release from SELP-47K hydrogels was evaluated. After purification from bacteria, plasmid DNA exists as a mixture of supercoiled, open-circular, and linear forms that can be resolved by gel electrophoresis. The treatment of supercoiled pRL-CMV with the *N.Bst*NI nicking enzyme resulted in loss of supercoiling and a transition to the predominantly open-circular form. Linear plasmids were prepared by treatment of pRL-CMV with the *Hind* III restriction enzyme, which cleaved both strands of the plasmid DNA generating linearized plasmid.

The percentage cumulative release of the different conformations of pRL-CMV over 28 days was on the order of linear > supercoiled > open circular (Fig. 3). The average effective diffusivity of the open circular

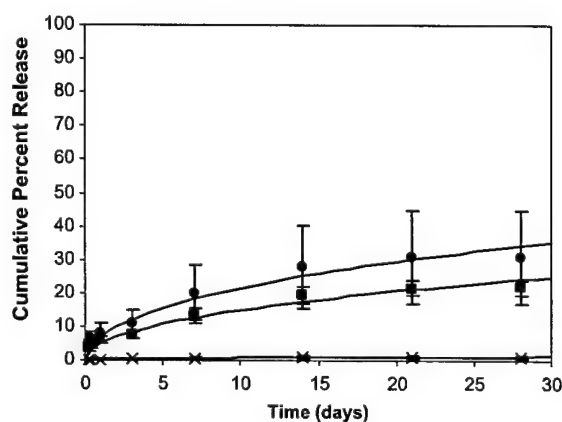


Fig. 3. Effect of pRL-CMV conformation on in vitro release from SELP-47K hydrogels: (●) linear, (■) supercoiled, (×) open-circular; (—) Theoretical release based on Eq. (1). Each data point represents the mean  $\pm$  S.D. for  $n=3$  samples.



conformation in the hydrogel was only  $1.96 \pm 0.83 \times 10^{-13} \text{ cm}^2/\text{s}$ . Moreover, the cumulative amount of open-circular pRL-CMV (4.08 kb) release was even less than that of the supercoiled plasmid pFB-ERV (11 kbp), even though the latter is almost three times larger in size.

The greater diffusivity of the linear and supercoiled plasmids relative to the open-circular plasmid arises from the different topologies of the three conformations. Previous studies have shown that linearized DNA diffuses through agarose hydrogels by a mechanism involving both free diffusion and reptation [28]. Transport of linearized DNA by reptation would be expected to increase its rate of release from SELP-47K hydrogels, as the pore size would be less restrictive to a semi-flexible, linearized molecule moving in a "snake-like" manner.

Supercoiled DNA, on the other hand, is known to have a larger diameter and shorter contour length than linear DNA [29]. The larger diameter of supercoiled DNA could possibly increase the contact between this molecule and the hydrogel network, leading to a rate of release less than that of linearized plasmid of the same molecular weight.

The essential lack of release of the open-circular form of pRL-CMV may be due to its impalement on the fibers that constitute the hydrogel matrix. Impalement of open-circular DNA on hydrogel matrices has previously been shown to occur during electrophoretic trapping in polyacrylamide and agarose hydrogels [30,31]. While no electric field was applied to the SELP-47K hydrogels, as occurs during electrophoretic trapping, it is likely that some of the physically cross-linked polymers extend through the open-circular plasmid preventing transport of the DNA. In addition, the potentially larger hydrodynamic volume of the open-circular form may further restrict its diffusion through the pores of SELP hydrogels. This possibility may be further investigated by determining the pore size distribution of the hydrogel and the hydrodynamic volume of the different plasmid DNA conformations.

### 3.3. Effect of hydrogel geometry on the release of plasmid DNA

To study the influence of hydrogel geometry on plasmid release, hydrogels containing pRL-CMV

were prepared in 1 and 3 ml syringes. Two geometries were evaluated, a "large disc", (from the 3 ml syringe) with a 4.3 mm radius and 2.0 mm height and a "cylinder", (from the 1 ml syringe) with a 2.3 mm radius and 5.1 mm height. The average volumes of the large disc and the cylinder hydrogels were 116 and 85 mm<sup>3</sup>, respectively; the difference due to minor variations in cutting the hydrogel. Over 28 days, the cumulative release of pRL-CMV from the large disc geometry was greater than that released from the cylindrical geometry (Fig. 4). The difference in the rate of release can be attributed to the difference in the surface-to-volume ratio of the two geometries. The large disc hydrogels had a surface area of 1.70 cm<sup>2</sup> while that of the cylindrical gels was 1.07 cm<sup>2</sup>, resulting in surface-to-volume ratios of 1.47 and 1.25 per cm, respectively. Hence, the rate of release from the large disc hydrogels is expected to be greater, as predicted by the application of Eq. (1) to the release data (Fig. 4).

### 3.4. Integrity and in vitro bioactivity of DNA encapsulated in SELP-47K hydrogels

DNA purified from the hydrogels after incubation in PBS at 37 °C for 28 days was found to remain structurally intact with regard to molecular weight and conformation by agarose gel electrophoresis (not shown). The in vitro bioactivity of the DNA purified

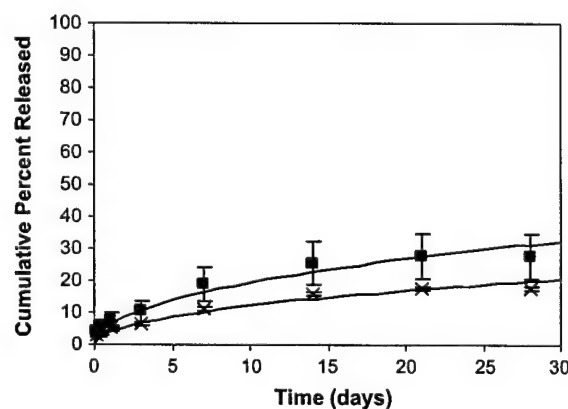


Fig. 4. Effect of SELP-47K hydrogel dimensions on in vitro release of pRL-CMV: (■) large disc, (×) cylindrical. (—) Theoretical release based on Eq. (1). Each data point represents the mean  $\pm$  S.D. for  $n=3$  samples.

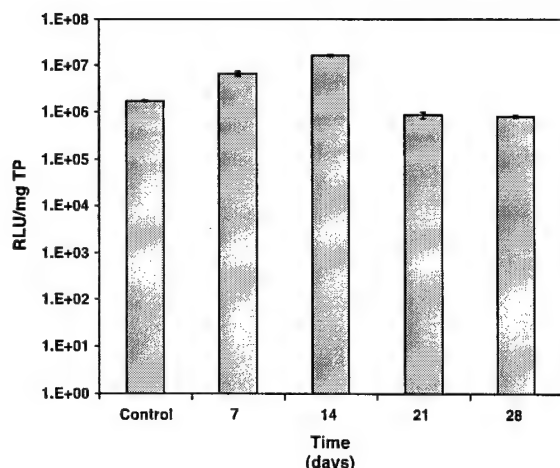


Fig. 5. In vitro bioactivity of plasmid DNA, expressed in relative luminescence units per mg TP content, after encapsulation in SELP-47K hydrogels for various periods of time. DNA encapsulated in the hydrogels retained bioactivity for at least 28 days. Control DNA was used to prepare the hydrogels prior to incubation at 37 °C. Each data point represents the mean  $\pm$  S.D. for  $n=3$  samples.

from the hydrogels was comparable to the control DNA used to prepare the hydrogels, indicating no significant loss in bioactivity after incubation in PBS at 37 °C for 28 days (Fig. 5).

### 3.5. In vivo delivery of pRL-CMV from silk-elastinlike hydrogels

The athymic nude mouse/MDA-MB-435 breast cancer model is an accepted model for studying human breast cancer [32–34]. This model has been used to study gene delivery to tumors experimentally induced in the mammary fat pad and in subcutaneous tissue, as well as to metastatic tumors [35,36]. The experimentally-induced subcutaneous tumor model used in this study provides relatively consistent access to tumors and surrounding tissue, in contrast to tumors in their native tissue. On the other hand, the physiology of subcutaneous tumors may somewhat differ from tumors that develop naturally in their respective tissue. Nevertheless, for the initial characterization studies, subcutaneous tumors provide relevant data for the evaluation of controlled release.

The injection of the polymeric matrices intratumorally and/or directly adjacent to the tumor was confirmed visually upon resection of most tumors.

Delivery of the *Renilla* luciferase plasmid from SELP-47K matrices resulted in significantly enhanced luciferase gene expression in tumors up to 21 days when compared to the delivery of plasmid without a polymer matrix. In particular, delivery of the plasmid from matrices containing 4 and 8 wt.% polymer resulted in greater gene expression, as compared to naked DNA until 21 days (Fig. 6). When DNA was delivered from 4 wt.% hydrogels, mean tumor transfection was 3.5 ( $P=0.014$ ), 154.2 ( $P=0.014$ ), 7.4 ( $P=0.016$ ), and 546.9 ( $P=0.008$ ) times greater at 3, 7, 14, and 21 days, respectively, than naked DNA. For 8 wt.% hydrogels, mean tumor transfection was 114.3 ( $P=0.033$ ), 14.5 ( $P=0.027$ ), 7.4 ( $P=0.016$ ), and 5.6 ( $P=0.013$ ) times greater at 3, 7, 21, and 21 days, respectively, than naked DNA. For 12 wt.% hydrogels, mean tumor transfection was 13.2 ( $P=0.015$ ) times greater at 3 days than naked DNA. At all other time points, the level of transfection in the tumor mediated by delivery from SELP hydrogels was similar to that mediated by naked DNA.

This data is consistent with sustained delivery from the 4 and 8 wt.% polymer matrices. The matrices composed of 12 wt.% polymer mediated transfection was significantly greater than naked DNA only at the 3 day time point. This is consistent with in vitro release data indicating entrapment of a fraction of

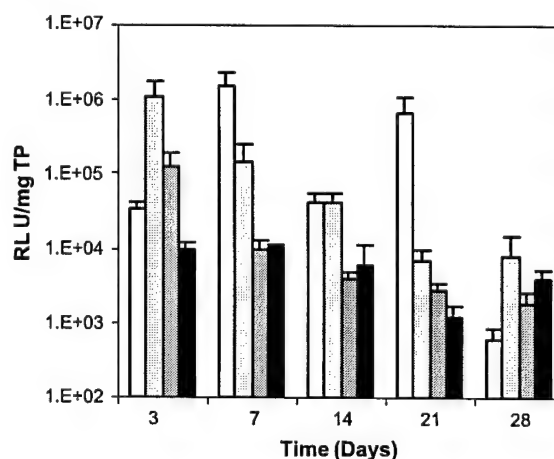


Fig. 6. Expression of *Renilla* luciferase in MDA-MD-435 tumors grown subcutaneously in athymic *nu/nu* mice, after intratumoral injection. Bars represent 4 wt.% polymer (white), 8 wt.% polymer (light gray), 12 wt.% polymer (dark gray), and naked DNA without polymer (black). Each bar represents the mean  $\pm$  standard error of the mean for  $n=4$  or  $n=5$  samples.



DNA within 12 wt.% matrices after gelation, limiting the release of DNA at later time points [17].

Differences in the level of tumor transfection mediated by polymeric matrices of different composition were not statistically significant until 7 days, when the transfection mediated by the 4 and 8 wt.% matrices were both greater than that mediated by the 12 wt.% matrix (Fig. 6). For 4 wt.% hydrogels, mean tumor transfection was 163.5 ( $P=0.018$ ), 11.2 ( $P=0.017$ ), and 233.2 ( $P=0.017$ ) times greater at 7, 14, and 21 days, respectively, than when DNA was delivered from 12 wt.% hydrogels. For 8 wt.% hydrogels, mean tumor transfection was 15.4 ( $P=0.035$ ) and 11.2 ( $P=0.008$ ) times greater at 7 and 14 days, respectively, than when DNA was delivered from 12 wt.% hydrogels. All other compositions and time points were similar.

Tumor transfection mediated by polymeric matrices generally showed a maximum at 3 or 7 days, then decreased to become approximately equivalent to naked DNA by 28 days. The decrease in transfection over time can possibly be attributed to a corresponding decrease in the release of plasmid due to entrapment, or depletion due to release and/or degradation. While not always significantly different by statistical criteria, the delivery of DNA from 4, 8, and 12 wt.% hydrogels resulted in a mean 142.4-, 28.7-, and 3.5-fold increase in transfection, respectively, compared with naked DNA over the entire 28-day period.

Luciferase expression in the skin approximately 1 cm around the tumor was evaluated to detect the transfection of cells in the surrounding tissue. With few exceptions, skin transfection was observed to be fairly similar between groups at all time points (Fig. 7). At 7 and 14 days, skin transfection mediated by the 8 wt.% matrix was 17.1 ( $P=0.007$ ) and 8.7 ( $P=0.033$ ) times that mediated by naked DNA, respectively. At 7 and 14 days, skin transfection mediated by the 8 wt.% matrix was 17.1 ( $P=0.014$ ) and 0.8 ( $P=0.008$ ) times that mediated by the 12 wt.% matrix. All other compositions and time points were similar.

The enhancement of delivery to the tumor was evaluated by comparing the levels of transfection in the tumors (Fig. 6) and skin (Fig. 7) at each time point and polymer concentration. At 3 days tumor transfection was significantly greater than skin transfection for all hydrogels, but not naked DNA. Specifically, tumor

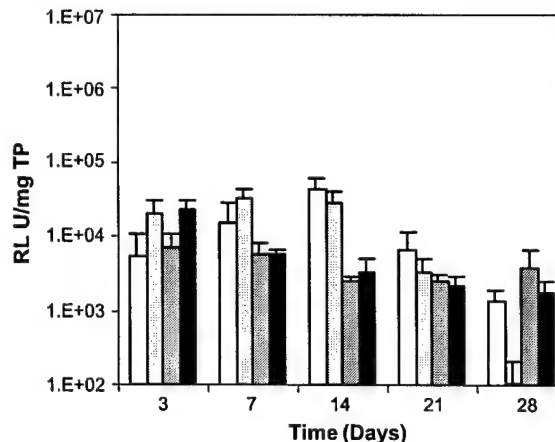


Fig. 7. Expression of *Renilla* luciferase in the skin directly surrounding ( $\sim 1$  cm) MDA-MD-435 tumors grown subcutaneously in athymic *nu/nu* mice. Bars represent 4 wt.% polymer (white), 8 wt.% polymer (light gray), 12 wt.% polymer (dark gray), and naked DNA without polymer (black). Each bar represents the mean  $\pm$  standard error of the mean for  $n=4$  or  $n=5$  samples.

transfection was 6.2 ( $P=0.018$ ), 55.4 ( $P=0.015$ ), and 18.4 ( $P=0.009$ ) times that in the skin at 3 days for 4, 8, and 12 wt.% hydrogels, respectively. For 4 wt.% hydrogels a difference between tumor and skin transfection was also detected at 7 and 21 days, where transfection was 101.6 ( $P=0.029$ ) and 100.7 ( $P=0.033$ ) times greater in the tumor, respectively. For naked DNA, a difference between tumor and skin transfection was detected only at 7 days, when tumor transfection was 1.7 ( $P=0.007$ ) times that in the skin. All other compositions and time points were similar. While statistically significant differences were not detected between all compositions, the mean tumor transfection was 42.0, 27.2, and 4.6 times greater than that in the skin for 4, 8, and 12 wt.% hydrogels, respectively, over the entire 28 day period. This is in contrast to a 1.3-fold difference between tumor and skin transfection for naked DNA. These results indicate that DNA delivered from the polymeric matrix enhanced transfection within the tumors, while the delivery of naked DNA lead to more equivalent levels of transfection in the tumors and skin.

Except for differences between 21 and 28 days ( $P=0.035$ ), transfection of tumors was similar for consecutive time points after administration of naked DNA. By contrast, the transfection mediated by polymeric matrices peaked between 3 and 7 days,

and then decreased to a level approximately equivalent to naked DNA by 28 days. Significant differences existed between time points for 12 wt.% hydrogels between 3 and 7 days ( $P=0.008$ ), 8 wt.% hydrogels between 14 and 21 days ( $P=0.015$ ), and 4 wt.% hydrogels between 21 and 28 days ( $P=0.008$ ). All other compositions and consecutive time points were similar.

Luciferase expression in the skin was fairly constant between all consecutive time points. Exceptions

occurred for naked DNA between 3 and 7 days ( $P=0.008$ ) and 8 wt.% hydrogels between 14 and 21 days ( $P=0.028$ ), and 21 and 28 days ( $P=0.015$ ). All other compositions and time points were similar.

### 3.6. Controlled delivery of adenovirus from SELP-47K hydrogels

Experiments were conducted to evaluate the in vitro release and bioactivity of adenoviral vectors

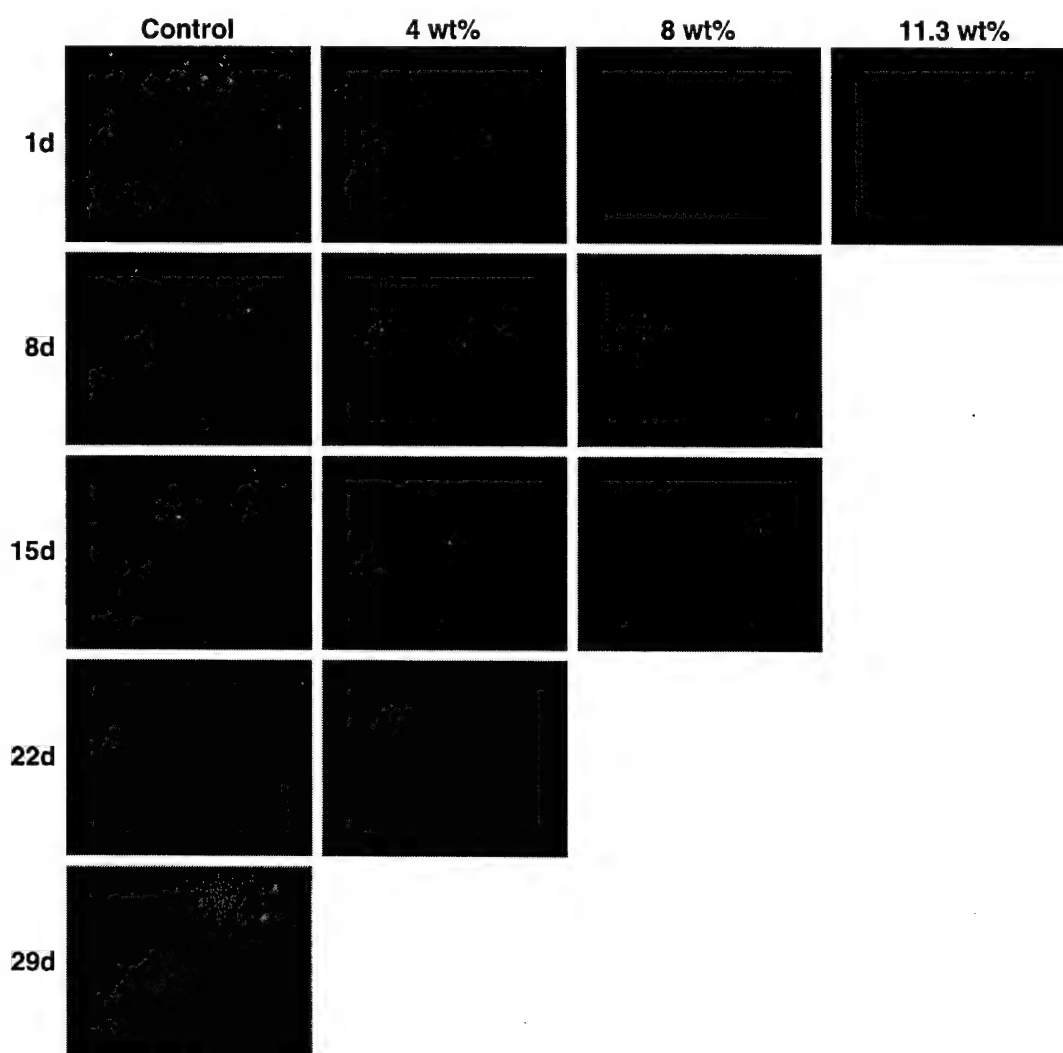


Fig. 8. Adenovirus release from SELP-47K and the corresponding bioactivity results. The percentage of polymer increases from left to right. The time of release of virus from gels used to transfect cells or control increases from top to bottom. The images are from fluorescent microscopy at  $40\times$  magnification. Bright spots represent individual cells transfected with AdGFP.

from SELP-47 K hydrogels. There was a clear qualitative relationship between the percentage of polymer and the number of transfected cells, indicating that the release of adenoviruses can be modulated by changing the composition of the matrix, to obtain a controlled release profile. Hydrogels composed of 4 wt.% polymer released bioactive adenovirus for at least 22 days (Fig. 8, Column 2), while less release was seen from 8 wt.% hydrogels (Fig. 8, Column 3) and virtually no release from 11.3 wt.% gels (Fig. 8, Column 4). This is probably due to the fact that the pore size of the 8 and 11.3 wt.% hydrogels significantly restricts diffusion of the adenovirus in the matrix. Another possibility, that the interaction of the polymer with adenoviral particles at higher polymer concentrations leads to decreased or no transduction, cannot be ruled out at this time.

#### 4. Conclusions

The conformational release studies showed that supercoiled DNA is the primary form released from the hydrogel indicating that in vivo transfection is most likely modulated by the supercoiled form of DNA. As expected, the size-dependent release studies indicate the potential to use SELP hydrogels to deliver plasmids that are much larger than the pRL-CMV (~4 kb) studied previously. The analysis of the influence of hydrogel geometry on release can be useful in determining where and how to inject SELP gene delivery systems. In vitro transfection studies indicate that encapsulated plasmid DNA and adenoviral particles are bioactive up to 28 and 22 days, respectively. The controlled delivery of adenoviral vectors from polymeric matrices may increase their therapeutic potential by increasing transfection efficiency and decreasing their immunogenicity and hepatotoxicity. Spatial localization of the delivered plasmid DNA in vivo demonstrates increased transfection efficiency in the tumor. These studies demonstrate the potential of recombinant silk-elastinlike hydrogels for controlled gene delivery. It must be noted, however, that despite the aforementioned advantages, the biological synthesis of polymers is limited by its high initial cost, restricted incorporation of unnatural amino acids, and unpredicted response of the biological expression system. Future

studies will include overcoming these challenges and tailor-making polymers using genetic engineering techniques for specific cancer gene therapy applications where localized control over release of viral and nonviral vectors are essential for improved efficacy and reduced toxicity.

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## Thermal Analysis of Water in Silk–Elastin-like Hydrogels by Differential Scanning Calorimetry

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### Introduction

Genetically engineered polymers are a class of protein-based materials that are synthesized using recombinant techniques.<sup>1,2</sup> This approach allows for the rational design and synthesis of amino acid-based macromolecules with precisely defined compositions, sequences, and molecular weights. Such a high level of control over polymeric architecture enables precise customization of the physico-chemical properties that are important for biomedical applications, such as biodegradation, stimuli-responsiveness, and biorecognition.<sup>2–4</sup> One class of genetically engineered polymers, the silk–elastin-like polymers (SELPs), has recently been evaluated as a matrix for the controlled delivery of drugs, proteins, DNA, and adenoviral particles.<sup>5–8</sup>

SELPs are hybrid block copolymers composed of alternating silk-like (Gly-Ala-Gly-Ala-Gly-Ser) and elastin-like (Gly-Val-Gly-Val-Pro) peptide blocks.<sup>1</sup> By combining the silk-like and elastin-like blocks in various ratios and sequences, it is possible to produce an assortment of block copolymers with diverse material properties. The solubility, material strength, immunogenicity, and biodegradation of SELPs can be controlled by varying the composition and sequence of the polymers.<sup>3</sup>

One SELP studied for its potential in the controlled release of bioactive agents is SELP-47K.<sup>5–7</sup> SELP-47K is a copolymer with four silk-like blocks and seven elastin-like blocks in its primary repetitive sequence (Figure 1, bold). It undergoes an irreversible sol-to-gel transition when transferred from room temperature to body temperature.<sup>5</sup> Hence, this polymer can form a depot for the controlled release of bioactive compounds, in situ, with minimally invasive spatial localization by injection through a needle or endoscope. Once localized, the matrix can release bioactive compounds while it degrades to its amino acid constituents.

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MDPVVLQRRDWENPGVTQLNRLAAHPFASDPMGAGS
GAGAGS[(GVGVVP)4GKGVVP(GVGVVP)3(GAGAGS)4]12(GV
GVP)4GKGVVP(GVGVVP)3(GAGAGS)2GAGAMDPGRYQDL
RSHHHHHH
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**Figure 1.** 884 amino acid SELP-47K sequence with a molecular weight of 69 814 Daltons. It is composed of a head and tail sequence, and a series of silk-like (GAGAGS) and elastin-like (GVGVVP) repeats (in bold). Abbreviation Key: A = alanine; D = aspartic acid; E = glutamic acid; F = phenylalanine; G = glycine; H = histidine; K = lysine; L = leucine; M = methionine; N = asparagine; R = arginine; P = proline; Q = glutamine; S = serine; T = threonine; V = valine; W = tryptophan; Y = tyrosine.

It is well-known that upon cooling a fraction of the water in hydrogels and other water–polymer systems does not freeze. This phenomenon has been documented for both chemically synthesized polymers<sup>9–12</sup> and proteins.<sup>13–16</sup> Non-freezable water has frequently been explained by hypothesizing the presence of a layer of “bound” water, which interacts with polymer chains by hydrogen bonding.<sup>9,10,17</sup> However, experiments show that nonfreezable solvent exists even when a nonpolar polymer is solvated with a nonpolar solvent, indicating that nonfreezable water may not be due to hydrogen-bonding alone.<sup>18</sup> Alternative explanations for this phenomenon have been proposed that rely on the rate of water diffusion during freezing, the hydrophobicity of the polymer, the glass transition temperature of the hydrogel, and the pore size of the network.<sup>13,19–21</sup>

Hence, although it is widely observed that nonfreezable water does exist, the nature of this water is not completely understood, and it is possible that more than one phenomenon may account for its existence. The importance in understanding the nature of this water lies in the potentially critical influence of polymer–solvent interactions on the microscopic and macroscopic properties of hydrogels, such as phase transitions, diffusion in the vicinity of polymer chains, the stability of drugs incorporated in hydrogels, and chromatographic separations.<sup>22–25</sup> The purpose of this note was to characterize the amount and, to the extent possible by differential scanning calorimetry, the nature of nonfreezable water in SELP-47K hydrogels.

### Experimental Section

**Materials.** The linear SELP protein copolymer SELP-47K (structure shown in Figure 1) was provided by Protein Polymer Technologies, Inc. (San Diego, CA) as a 12 wt % aqueous solution in 3 mL syringes, frozen at –80 °C. Hermetic aluminum pans, indium, and sapphire were purchased from TA Instruments (New Castle, DE). Phosphorus pentoxide was purchased from Sigma-Aldrich (St. Louis, MO).

**Preparation of Hydrogels for Differential Scanning Calorimetry.** SELP-47K solutions (12 wt %) were thawed by placing a syringe in room-temperature water for five minutes. The polymer solution was dispensed into 1 mL



## B Notes

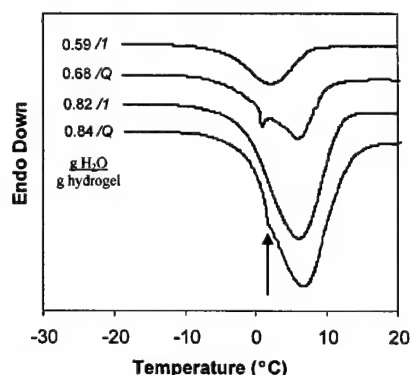
## Biomacromolecules

syringes, used as molds, which were sealed and placed upright in an incubator adjusted to 37 °C for 24 h, to allow gelation. The hydrogels were ejected from the syringe in cylindrical form and cut into disks of appropriate size with a razor blade. Disks were incubated at 37 °C with shaking (120 rpm) for 5 days in one liter of frequently replaced purified, deionized water to remove the polymer soluble fraction and buffer salts. After the five-day wash-out period, hydrogels were stored in purified, deionized water at 4 °C.

To prepare samples for DSC, disks of appropriate size to achieve a sample weight of approximately 10 mg after drying to the respective water content were removed from storage, carefully dried with a lint-free tissue to remove surface water, and placed in preweighed, hermetic aluminum DSC pans. To prevent heat flow anomalies from the reaction of water with the aluminum pan, pans were boiled in purified, deionized water for 1 h, and dried thoroughly prior to encapsulation of the hydrogels. This method induces a thin layer of aluminum oxide formation on the pans that prevents further reaction of water and the aluminum surface. Samples were sealed in the pans and allowed to equilibrate overnight, at room temperature prior to analysis. Sample weight was checked prior to and after analysis to ensure the integrity of the hermetic seal. To obtain samples with water contents less than ~90 wt %, hydrogels were dried in the open sample pan at 37 °C prior to encapsulation.

**Differential Scanning Calorimetry.** DSC was performed on a TA Instruments model 2920 differential scanning calorimeter. The temperature calibration was performed with purified, deionized water and indium. Heat flow was calibrated with indium. Heat capacity was calibrated with sapphire. The sample cell was purged with helium during cooling and nitrogen during heating, each at a flow rate of 50 mL/min. For heat of fusion ( $\Delta H_f$ ) measurements, hydrogels were cooled to -70 °C and heated at a rate of 2.5 °C/min to 60 °C. Heat capacity ( $C_p$ ) measurements were obtained by quasi-isothermal modulated differential scanning calorimetry (QIT-MDSC), with a modulation amplitude of  $\pm 0.5$  °C and a period of 80 s. Unlike scanning DSC, QIT-MDSC allows the measurement of heat capacities under quasi-equilibrium conditions. To minimize artifacts from differences in aluminum pans, sample pan weights were matched with reference pans to within  $\pm 0.1$  mg.

**Calculation of the Amounts of Freezable and Non-freezable Water.** Total water content was determined gravimetrically, by weighing the samples before and after encapsulation, equilibration, analysis, and drying on a balance accurate to  $\pm 100$   $\mu$ g. To determine dry weight, a pinhole was made in the top of the aluminum pan. The sample was dried for at least 5 days over phosphorus pentoxide, followed by 24 h under vacuum at 25 °C. The hydrogels did not lose detectable weight upon further drying. The amount of freezable water for each sample was determined by integration of the endothermic transition due to the melting of water, as measured by DSC and assuming a heat of fusion of 334 J/g. The amount of freezable water was subtracted from the total water content, determined gravimetrically, to obtain the nonfreezable water content.



**Figure 2.** Differential scanning calorimetry heat flow data showing the melting of water in SELP-47K hydrogels as a function of hydration (g H<sub>2</sub>O/g hydrogel). Q denotes quench cooling (rapid, rate uncontrolled); 1 denotes a cooling rate of 1 °C/min. The minor peak present in some samples (0.68/Q, 0.84/Q) disappeared when the cooling rate was changed from quench to 1 °C/min (0.59/1, 0.82/1).

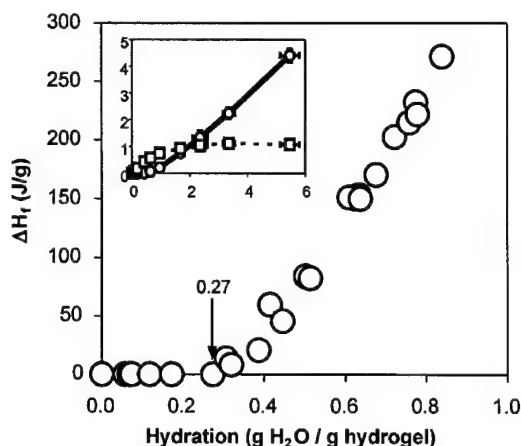
## Results and Discussion

Typical DSC curves for hydrogels of various water contents are shown in Figure 2. Due to the presence of dual peaks while heating some samples, the calorimeter was calibrated so that the melting temperature occurred at the onset (not peak) of the transition. Due to the fact that no thermal transitions were observed outside of the -20 to +20 °C temperature range, only this temperature range is presented. As expected, the area of the endothermic transition decreases with decreasing water content.

Polymers composed of exclusively elastin-like (GVGVP) repeats (elastin-like polymers, ELPs), and SELPs containing one silk unit per monomer repeat, undergo a phase transition in response to changes in temperature, pH, ionic strength, and polymer concentration.<sup>26,27</sup> The mechanism of this transition in ELPs has been investigated both experimentally and computationally, and is typically explained by the presence of water of hydrophobic hydration, which surrounds hydrophobic amino acid residues.<sup>28,29</sup> This water is thought to have characteristics that are different from bulk water, due to the fact that it cannot form hydrogen bonds with hydrophobic residues.

Previous DSC studies on soluble ELPs have shown thermal transitions in the temperature range of 30–50 °C that have been attributed to the release of the water of hydrophobic hydration, upon conversion of the ELP from an extended to a collapsed  $\beta$ -spiral.<sup>30</sup> We did not observe any thermal transitions in SELP-47K hydrogels (containing four silk repeats per monomer) that were attributable to such a transition. A possible explanation for the lack of a thermally induced elastin phase transition in SELP-47K hydrogels is that the elastin-like blocks in this polymer are conformationally constrained by the self-assembly of the silk-like blocks. Consistent with this explanation, previous studies have shown that SELP-47K hydrogels do not exhibit changes in bulk properties (e.g., swelling) in response to changes in temperature, pH, and ionic strength.<sup>31</sup> By contrast, chemically cross-linked hydrogels made from ELPs are known to exhibit responsiveness to these stimuli.<sup>32</sup>

The appearance of a second, smaller peak (Figure 2, Fraction H<sub>2</sub>O = 0.84/Q, 0.68/Q) with a temperature less than



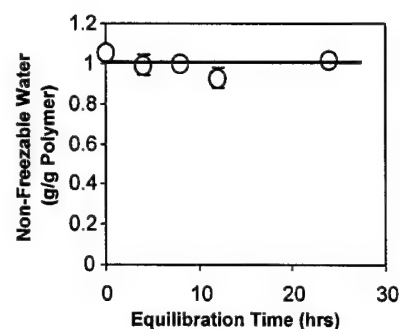
**Figure 3.** Heat of fusion of water in SELP-47K hydrogels as a function of hydration (g H<sub>2</sub>O/g hydrogel). DSC did not detect any water in the hydrogels until approximately 27 wt %. Above this water content, the heat of fusion increased linearly ( $r^2 = 0.9831$ ). Inset: Effect of hydration (g H<sub>2</sub>O/g polymer) (abscissa) on the amounts of nonfreezable (□) and freezable (○) water (ordinate). Nonfreezable water increased to a level of ~1 g/g of polymer at a total level of hydration of approximately 2 g/g of polymer. Each data point is the mean  $\pm$  standard deviation of triplicates.

that of the primary peak has been cited as evidence for the existence of an intermediate form of weakly bound water.<sup>33</sup> However, it has also been shown that for at least some hydrogels this peak does not represent the existence of intermediate water, but rather may arise from the presence of a metastable form of water.<sup>34</sup> Consistent with this explanation, when we decreased the cooling rate from quench (rapid) speed to 1 °C/min, this peak was no longer observed (Figure 2, Fraction H<sub>2</sub>O = 0.82/1, 0.59/1). It appears that this minor peak, at least in SELP-47K hydrogels, is an artifact of the cooling rate. Regardless of the cooling rate, crystallization of water always occurred in a single, sharp exothermic peak (not shown). This is in contrast to poly(2-hydroxyethyl methacrylate) hydrogels, in which two peaks have been observed during cooling.<sup>35</sup>

Notably, the minor endothermic peak (Figure 2) could also result from a fraction of the water being trapped in the pores of the hydrogel.<sup>13</sup> In the case of SELP-47K, the slower cooling rate (1 °C/min vs quench) may allow more time for water to diffuse out of pores and associate with ice crystals during cooling, and before becoming immobilized in the frozen glassy matrix.<sup>21</sup>

Unlike the melting of pure bulk water, the transitions in Figure 2 appear to occur over a very broad temperature range (~25 °C). Although we cannot conclusively say why this occurs at present, it is possible that these transitions are the result of the melting of water clusters, segregated by the hydrogel network, and not bulk water. Another possible explanation is that some of the energy input during this transition is being absorbed for the purposes of the endothermic mixing of the melted water and polymer chains.

Consistent with reports on many other water-polymer systems, a fraction of the water contained in SELP-47K hydrogels was nonfreezable.<sup>9-13,16,17</sup> Specifically, no detectable freezing or melting occurred until a water content of approximately 27 wt % (Figure 3). The inset in Figure 3



**Figure 4.** Effect of equilibration time at -15 °C on the amount of nonfreezable water in hydrogels with 87 wt % water. Nonfreezable water content was found to be largely independent of the equilibration time. Each data point is the mean  $\pm$  standard deviation of triplicates.

shows the amounts of freezable and nonfreezable water in the hydrogels at various levels of hydration, calculated from the heat of fusion data. The trends in this relationship are qualitatively consistent with observations of poly (methyl methacrylate) (PMMA) hydrogels, although the maximum amount of nonfreezable water in SELP-47K hydrogels (Figure 3, inset) is approximately 4–5 times greater.<sup>12</sup> However, the amounts of nonfreezable water should be interpreted as estimates, as it has been proposed that the heat of fusion of freezable water in polymer/water systems may not be constant and that the value of 334 J/g is a maximum, leading to a possible overestimation of the nonfreezable water content.<sup>36</sup> For this reason, the intercept of the  $\Delta H_f$  versus hydration line (Figure 3) may be a more accurate estimation of nonfreezable water content.

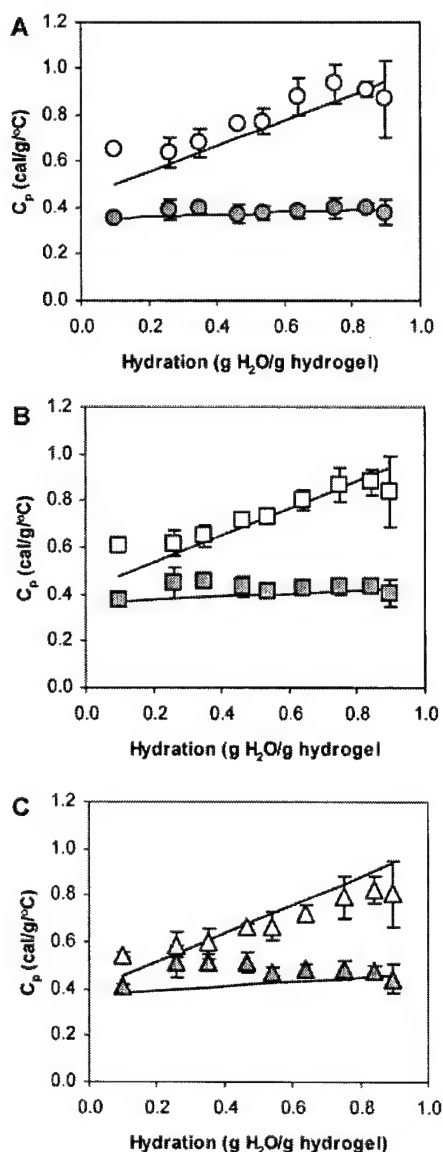
The amount of nonfreezable water in methacrylic acid-based hydrogels substantially decreases when the hydrogels are equilibrated at -15 °C for up to 14 h.<sup>11</sup> This kinetic effect is explained by considering the restricted diffusion of water in hydrogels and the transition of the hydrogel from a rubbery to a glassy state during freezing. Equilibrating the hydrogels for extended periods of time allows water molecules more time to diffuse and associate with ice crystals. We did not observe such a kinetic effect in SELP-47K hydrogels, even after equilibration for up to 24 h (Figure 4). The lack of a kinetic effect in SELP-47K hydrogels and the relatively large amount of nonfreezable water may be related to the hydrophobicity of this copolymer.

Recently, it was reported that increasing the fraction of a hydrophobic polymer in an interpenetrating network resulted in an increase in the nonfreezable water content.<sup>20</sup> It was proposed that the hydrophobic polymer presents a diffusion barrier to water within the hydrogel. Hydrophobically modified poly(acrylic acid)-based hydrogels exhibit similar behavior.<sup>37</sup> SELP-47K contains a large fraction of hydrophobic amino acids, primarily valine (22.4%) and alanine (12.2%), raising the possibility that this polymer could behave similarly.

The term *bound water* implies a different thermodynamic state of water in which restricted molecular mobility is inherent, either due to a perturbation in hydrogen bonding between water molecules themselves or between water molecules and polymer chains.<sup>12,14</sup> To estimate the molecular mobility of water in SELP-47K hydrogels relative to normal

## D Notes

## Biomacromolecules

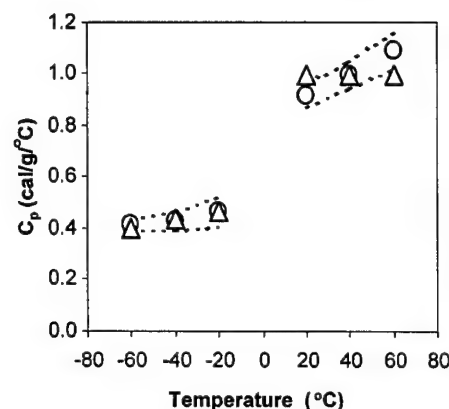


**Figure 5.** Effect of hydrogel hydration (g H<sub>2</sub>O/g polymer) on  $C_p$  at (A) 60 °C (○) and -60 °C (●); (B) 40 °C (□) and -40 °C (■); and (C) 20 °C (△) and -20 °C (▲). The solid lines near each set of data points are calculated heat capacities determined, using eq 1, based on the measured  $C_p$  of polymer and literature values for water.<sup>39,40</sup> Each data point is the mean  $\pm$  standard deviation ( $n \geq 3$ ).

water, the heat capacity of the hydrogels was measured at various temperatures and levels of hydration. A substantial decrease in the molecular mobility of nonfreezable water (i.e., binding) should result in a decreased partial specific heat capacity of this water in the hydrogel relative to what would be expected with unbound or normal water.<sup>12,14</sup> As shown in Figure 5, the heat capacity of the hydrogels at six temperatures and nine water contents was described well by eq 1:

$$C_{p(\text{hydrogel})} = f_{\text{water}} C_{p(\text{water})} + f_{\text{polymer}} C_{p(\text{polymer})} \quad (1)$$

where  $C_p$  is the heat capacity of the hydrogel, water, or polymer, and  $f$  is the fraction (g/g hydrogel) of the water or polymer. This relationship can be expected to hold for a two-



**Figure 6.** Calculated (○) partial heat capacity of water in SELP-47K hydrogels, at various temperatures, versus accepted values in the literature (△). Dotted lines are 95% confidence interval of data set with 45 data points for each temperature.

phase system, composed of water and polymer. If the hydrogel contains two thermodynamically distinct phases of water (i.e., one bound and one free) with different heat capacities, eq 1 would be modified to include a third term, describing the fraction of and heat capacity of the bound water.

At temperatures below freezing and in the water content range of approximately 0.2–0.3 g/g hydrogel, there appears to be a positive deviation from the predictions of eq 1 (Figure 5). Since SELP-47K contains elastin-like repeats, it is possible that the observed departure from the predictions of eq 1, in this range of hydration, is due to a glass transition similar to that observed by Kakivaya and Hoeve.<sup>38</sup> Furthermore, at temperatures above 0 °C, there appears to be a positive deviation from the predictions of eq 1 at a water content of 0.1 g/g hydrogel. The reason for this deviation is unclear at this time, though it is possible that the polymer undergoes a transition as it is dried and more polymer–polymer contacts develop.

Using eq 1 and measured values of polymer heat capacity, the partial specific heat capacity of water within the hydrogels was calculated (Figure 6).<sup>14</sup> The calculated values closely correspond with literature values for bulk water, indicating that it is unlikely that all of the nonfreezable water in SELP-47K hydrogels is bound.

## Conclusions

DSC studies showed that up to 27 wt % nonfreezable water exists in SELP-47K hydrogels. The presence of a second, minor peak upon melting of water appears to be an artifact of the cooling rate rather than the existence of an intermediately bound phase of water. Within a 24 h period, the amount of nonfreezable water was not substantially affected by equilibration time. Heat capacity measurements at various temperatures and levels of hydration indicate that water in SELP-47K hydrogels has a partial specific heat capacity similar to that of normal water.

Using other hydrogel-forming polymers and proteins as a comparison, the existence of nonfreezable water in SELP-47K hydrogels is proposed to arise from the hydrophobicity of the polymer and/or the presence of nanocavities that may



be created during self-assembly. The former has previously been observed in interpenetrating hydrogel networks composed of poly(ethylacrylate) and poly(hydroxyethylacrylate).<sup>20</sup> The latter has been observed in protein-based materials, including collagen and gelatin.<sup>13,14</sup> Although the calorimetric data presented in this note indicate that it is unlikely that all nonfreezable water is bound, the indirect nature of calorimetric measurements prevent conclusions about the primary origins of nonfreezable water. Further insight into the nature of this water may be obtained by pulse field gradient NMR experiments.

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Review

## Genetically engineered polymers: status and prospects for controlled release

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### Abstract

Genetic engineering methodology has enabled the synthesis of protein-based polymers with precisely controlled structures. Protein-based polymers have well-defined molecular weights, monomer compositions, sequences and stereochemistries. The incorporation of tailor-made motifs at specified locations by recombinant techniques allows the formation of hydrogels, sensitivity to environmental stimuli, complexation with drugs and nucleic acids, biorecognition and biodegradation. Accordingly, a special interest has emerged for the use of protein-based polymers for controlled drug and gene delivery, tissue engineering and other biomedical applications. This article is a review of genetically engineered polymers, their physicochemical characteristics, synthetic strategies used to produce them and their biomedical applications with emphasis on controlled release.

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**Keywords:** Genetic engineering; Protein-based polymers; Stimuli-sensitive; Drug delivery; Gene delivery

### 1. Introduction

The nature and biological fate of polymers used in biomedical applications, such as implants and drug delivery systems, depends on their molecular architecture. The molecular weight, composition, sequence and stereochemistry of chemically synthesized polymers are usually heterogeneous and de-

finied in terms of statistical distributions [1]. Progress in recombinant DNA technology has enabled the synthesis of genetically engineered protein-based polymers with precisely defined molecular weights, compositions, sequences and stereochemistries [1–6]. Such detailed control over the molecular structure of a polymer allows similarly fine control over its physicochemical characteristics and biological fate [7–9]. It enables the construction of new tailor-made polymeric biomaterials with improved properties important for controlled drug delivery, such as better-defined gelation kinetics, biorecognition, biodegradation and stimuli-sensitivity.

A *genetically engineered protein-based polymer* (hereafter referred to as a *genetically engineered polymer* or *protein-based polymer*) is a polymer

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consisting of peptide sequence repeats, where each repeating unit can be composed of as few as two or as many as hundreds of amino acid residues, and may recur from a few to hundreds of times [10]. The key distinction that separates genetically engineered polymers from *poly(amino acid)s* and *sequential polypeptides* is that they are synthesized by recombinant techniques. Poly(amino acid)s are homo- or copolymers resulting from chemical polymerization of a single amino acid or a mixture of amino acids, respectively. Sequential polypeptides are produced by polymerization of short pre-synthesized peptide sequences whose molecular weight and possibly order of peptide blocks are not uniform. In contrast to chemically synthesized poly(amino acid)s and sequential polypeptides, the entire amino acid sequence of genetically engineered polymers is controlled at the DNA-level, leading to polymers with precisely defined, and potentially quite complex, sequences and structures [11]. Protein-based polymers can be designed to incorporate a variety of functionalities, including responsiveness to microenvironmental stimuli, controlled biodegradation and the presentation of informational motifs for cellular and subcellular interactions.

In this article, we will review the different classes of genetically engineered polymers and their physicochemical properties. Various biosynthetic strategies that have been used to produce these polymers will be discussed and current biomedical applications of these biomaterials will be reviewed, with emphasis on controlled release.

## 2. Classes of genetically engineered polymers

### 2.1. Elastin-like polymers

Elastin is an extracellular matrix protein consisting of several repetitive amino acid sequences, including VPGVG, APGVGV, VPGFGVGAG and VPGG [12,13]. Studies on chemically synthesized and genetically engineered elastin-like polymers (ELPs) have demonstrated that these constructs are soluble in aqueous medium, below their inverse transition temperature ( $T_i$ ) [10,14,15]. When the temperature is raised above the  $T_i$  ( $\sim 2^\circ\text{C}$  range), ELPs undergo a sharp phase transition leading to

desolvation and aggregation [10]. For example, polypeptides composed of the pentad sequence VPGVG undergo a temperature-induced transition from an extended to a collapsed  $\beta$ -spiral structure with three pentad units per type II  $\beta$ -turn. This structural change results in molecular aggregation that provides a regular arrangement of elastin molecules for crosslinking, to form insoluble elastic fibers in vivo [16]. Aggregates of elastin have sufficient mass to be removed from a solvent by centrifugation. As the phase transition is reversible, elastin molecules are completely resolubilized when the temperature is lowered below their  $T_i$  [10]. Studies on genetically engineered elastin-like polymers show that  $T_i$  is a function of polymer concentration [17], chain length [18] and amino acid composition [5]. In addition to temperature change,  $T_i$  can also be induced by changes in pressure, ionic strength and pH [10,16].

Most genetically engineered ELPs have been based on the repetitive pentapeptide motif VPGXG (where the “guest residue” X is any amino acid except Pro). Non-conserved amino acid substitutions can be incorporated in the fourth position of the pentapeptide without significant disruption in the secondary  $\beta$ -turn structure of the peptide. Such substitution can be employed to shift  $T_i$  in a reproducible and predictable manner for different applications [5,10]. Numerous applications of ELPs in biotechnology and medicine have been proposed [17–22]. Applications of these polymers in drug delivery are reviewed in Section 4.1.

The phase transition of ELPs, can be characterized by monitoring the absorbance of ELP solutions as a function of temperature. Reversibility of the transition is examined by incremental heating of ELP solutions followed by incremental cooling. The  $T_i$  is generally defined from the heating profile as the temperature of 50% maximum turbidity [10]. Aggregation of ELP fusion proteins has been investigated as a function of temperature by dynamic light scattering (DLS) [23]. The appearance of large aggregates closely coincides with the rise in turbidity observed by UV spectrophotometric techniques.

The mechanism of the ELP solubility transition has been proposed to involve reversible hydration of the hydrophobic residues of the polymer chain [10]. In solution, the hydrophobic residues are thought to

be surrounded by ordered water (water of hydrophobic hydration). As the temperature rises, this water becomes less-ordered bulk water, and the polymer collapses and aggregates by hydrophobic self-assembly. This mechanism has been supported by dielectric relaxation experiments [24] and computational simulations [25,26]. The inverse temperature transition has also been studied by differential scanning calorimetry [10]. Reversibility of physical changes in stimuli-sensitive biomaterials may be desirable for applications such as controlled drug delivery from polymer–drug conjugates [20], temporary functional scaffoldings for tissue restoration [21] and reversible cell culture coatings [27].

ELPs induce little or no immunogenic response in vivo [28]. Nanoparticle formulations of protein-based polymers composed of the tetrapeptide sequence (APGG) present a poly(ethylene glycol) (PEG)-like biocompatible interface, which allows efficient protection of a hydrophobic core from recognition by immune system and prevents clearance by macrophages [28,29].

## 2.2. Silk-like polymers

Silks are fibrous proteins composed of repetitive sequences of both crystalline and amorphous domains [30]. Silks are naturally produced by spiders (e.g. *Nephila clavipes*) and other insects, such as the silk worm *Bombyx mori*. Each species of silk-producing organism produces silk(s) with a different amino acid composition and mechanical properties that can be related to its function. The primary amino acid components of silk proteins are glycine, alanine and other short chain amino acids. Solid-state nuclear magnetic resonance (NMR) analysis has shown that alanine-rich domains, such as the poly alanine regions present in *N. clavipes* spider silk, form antiparallel  $\beta$ -sheet crystals by hydrogen bonding and hydrophobic interactions, while other regions of the protein form semi-crystalline or amorphous domains [31,32]. The small size of alanine is thought to stabilize the  $\beta$ -sheet crystal structure by allowing close packing of interlocking aliphatic side chains [33,34].

The crystalline region from *B. mori* fibroin contains a 59-amino acid repeat [GAGAGSGAAG[SGAGAG]<sub>8</sub>Y], with an approximate 3:2:1 ratio of glycine

to alanine to serine [35]. By contrast, *N. clavipes* produces two types of silk, called *N. clavipes* major ampullate glands 1 and 2 (NCMAG1 and NCMAG2, or spidroin 1 and spidroin 2) [36]. NCMAG1 consists of the repetitive sequence [GGAGQGGYGGGLGSQ-GAGRGGLGGQGGAG] [37–39], while NCMAG2 contains the repetitive sequence [GPGGYGGPGQ-QGPGGYAPGQQPSGPGS] [38,40,41]. Both repetitive sequences are interspersed with poly(alanine) regions that are thought to play a role in  $\beta$ -sheet formation [38,40]. These three silk sequences have been used as the basis for the design of several genetically engineered silk-like polymers (SLPs) [2, 42–44].

The crystalline properties of genetically engineered SLPs can be tailored by the periodic incorporation of amino acids that cannot participate in the  $\beta$ -sheet structure of the polypeptide. For example, the incorporation of a GAAGY pentad sequence, with a bulky tyrosine residue (Y) into [GAGAGS]<sub>9</sub>, lowers the crystallinity of this SLP [2].

While recombinant silk proteins have been successfully synthesized, their low aqueous solubility has been a significant barrier to their characterization and use in biomedical applications [45,46]. The extremely low solubility of recombinant silks can be attributed to the previously described inter-molecular hydrogen-bond formation [45]. Spider silks are insoluble in most solvents including water, dilute acid and alkali [47]. They may be hydrolyzed by concentrated sulfuric acid [48] and solubilized by 9 M lithium bromide and concentrated formic acid without hydrolysis [39].

To enhance the aqueous solubility of recombinant silks, several approaches have been investigated. One method depends on the inclusion of encoded triggers that regulate self-assembly of the silk, thus improving control over solubility [49–54]. For example, enzymatic phosphorylation and dephosphorylation has been employed to regulate the formation of  $\beta$ -sheet secondary structure in genetically engineered silks. Phosphorylation of recombinant silks decreases the  $\beta$ -sheet content, through a combination of steric hindrance and charge. The phosphorylated recombinant silk has a solubility that is approximately four-fold greater than the unphosphorylated form. Self-assembly can then be triggered by the introduction of a phosphatase into the solution [51].

Another approach to control the solution and solid-state behavior of  $\beta$ -sheet forming proteins, such as silk, is by utilizing the redox state of methionine. Recombinant drag-line silks were designed with methionine residues flanking a region encoding five alanine residues [53]. The assembly of the protein was regulated by the redox state of the methionyl side chain. In the reduced state, the methionines (hydrophobic and relatively small) permit normal poly alanine hydrophobic domain interactions, leading to  $\beta$ -sheet formation. In the oxidized state, self-assembly is inhibited by a combination of increased bulkiness due to disruption of  $\beta$ -sheet formation and hydrophilicity at the sulfoxide side chain [49,53].

Improved solubility of recombinant silks can also be achieved by construction of chimeric proteins [55]. Insoluble  $\beta$ -sheet-forming poly alanine has been combined with  $[GAGXGS]_x$ , where X is tyrosine or valine. This motif is derived from *B. mori* and enhances the solubility of the silk in water [56]. Improved solubility of the chimeric protein is due to the formation of  $\alpha$ -helical structures [55].

Teule et al. [57] investigated the importance of the presence of alanine motifs, and their role in the mechanical properties of SLPs, by synthesis of recombinant spidroin 1-like proteins with varying amounts of alanine. Results showed that alanine motifs are important for intracellular accumulation and stability of secreted spidroin 1-like proteins in the culture medium. The effect of the incorporation of a customized collagen sequence on the solubility of these silks was also studied [57]. Analysis of the expression of collagen-spidroin 1-like copolymer genes in the yeast *Pichia pastoris* showed that only copolymers rich in alanine motifs can be successfully produced and secreted from yeast as dimers. The results also showed that the incorporation of collagen improved the solubility of the expressed spidroin 1-like proteins.

Recent studies have examined the feasibility of using mammalian cells for the expression of recombinant spider silk proteins [58]. High molecular weight recombinant spider silk proteins were produced that were secreted into the medium and purified by precipitation with ammonium sulfate. Silk-like materials have also been made by transgenic goats containing spider silk proteins, which are purified and spun into fibres for use in a variety of applications [59].

### 2.3. Silk–elastinlike block copolymers

The family of silk–elastinlike block copolymers (SELPs) constitutes a well-characterized example of the control over physicochemical characteristics that can be achieved by variations in copolymer composition and sequence, using genetic engineering techniques [60,61]. The structure of these polymers can be generally defined by  $\{[S]_m[E]_n\}_o$ , where S represents a silk-like block consisting primarily of repeats of GAGAGS, derived from silk fibroin [62], and E is an elastin-like block with the general amino acid sequence VPGVG, derived from mammalian elastin [63]. However, these blocks may be substituted with other amino acids at some positions without major alteration in the fundamental properties of the SELP. As discussed previously, polymers composed of only silk-like blocks have very low aqueous solubility. Periodic incorporation of elastin-like blocks increases the solubility of silk-like polymers by reducing their total crystallinity [2]. It has been shown that increasing the number of consecutive silk-like blocks within a silk–elastinlike copolymer increases the rate of gelation and decreases the rate of bioresorption of the polymer [11,64]. These studies highlight the effect of polymeric sequence, a variable that is difficult to define in chemically synthesized polymers, on characteristics that are highly relevant for controlled drug delivery.

SELP solutions of appropriate composition and concentration are fluid at room temperature and can be injected through fine gauge hypodermic needles. With time, they form firm yet pliable hydrogels that are no longer water soluble [65]. The rate of gelation is influenced by the composition and concentration of the copolymer, and the environmental conditions, including temperature, ionic strength and pH. The gel-forming polymers crosslink physically. Therefore, no chemical modifications occur to the polymer structure or to the bioactive agents (small molecular weight drugs, nucleic acids, proteins, etc.) that might be incorporated in the polymer solution. Release of solutes from these hydrogels is dependent upon the molecular weight, charge and solubility of the solute, its diffusion through the polymeric matrix, the composition of the SELP forming the hydrogel, and the conditions under which release takes place, such as the pH and ionic strength of the release medium [64,66,67].

SELPs containing one silk unit per monomer repeat do not form hydrogels but, similar to ELPs, undergo a phase transition in aqueous medium in response to changes in temperature, pH, ionic strength and polymer concentration [68]. The mechanism of this transition is explained by the presence of water of hydrophobic hydration, which surrounds hydrophobic amino acid residues of elastin [24]. This water is thought to have characteristics that are different from bulk water, due to the fact that it cannot form hydrogen bonds with hydrophobic residues.

Nagarsekar et al. [68,69] showed that the amino acid sequence and polymer chain length of silk-elastinlike polymers with one silk unit in the monomer repeat (structures shown in Fig. 1, Panel A) can be tailored to control sensitivity to environmental stimuli. The replacement of the nonpolar valine residue in (17V)<sub>11</sub> by the ionizable glutamic acid residue (17E)<sub>11</sub> resulted in a reversible solubility transition sensitive to pH and ionic strength. (17E)<sub>11</sub> is a more hydrophilic polymer with a higher  $T_i$  than (17V)<sub>11</sub> (Fig. 1, Panel B), as ionizable glutamic acid residues are solvated by water of ionic hydration and water of polar hydration that require higher energy to separate than nonionic valine residues [68]. The phase transition reversibility occurs probably because the waters of hydrophobic hydration reorganize themselves on the polymer upon cooling, and the rehydrated polymer goes back into solution. For a given ionic strength,  $T_i$  of (17E)<sub>11</sub> and (17E)<sub>16</sub> (polymers with the same monomer structures but different molecular weights) increased as the pH increased from 3.0 to 6.0 [68,69]. This correlates with the degree of ionization of the glutamic acid side chains in the same pH range [24,70]. On the other hand, polymer (17V)<sub>11</sub>, without the ionizable glutamic acid residue in the monomer repeat, did not show appreciable sensitivity to pH [68].

In addition to the influence of monomer composition, increasing SELP chain length resulted in a lowering of  $T_i$  and reduction in sensitivity to changes in ionic strength and pH (Fig. 1, Panel C). SELP (17E)<sub>11</sub>, with 11 repeats of the glutamic acid-containing monomer, showed a higher transition temperature than (17E)<sub>16</sub>, a polymer with 16 repeats of the same monomer [68]. This phenomenon could be due to more intra-polymer interactions in the longer polymer chain. Together, these results indicate that

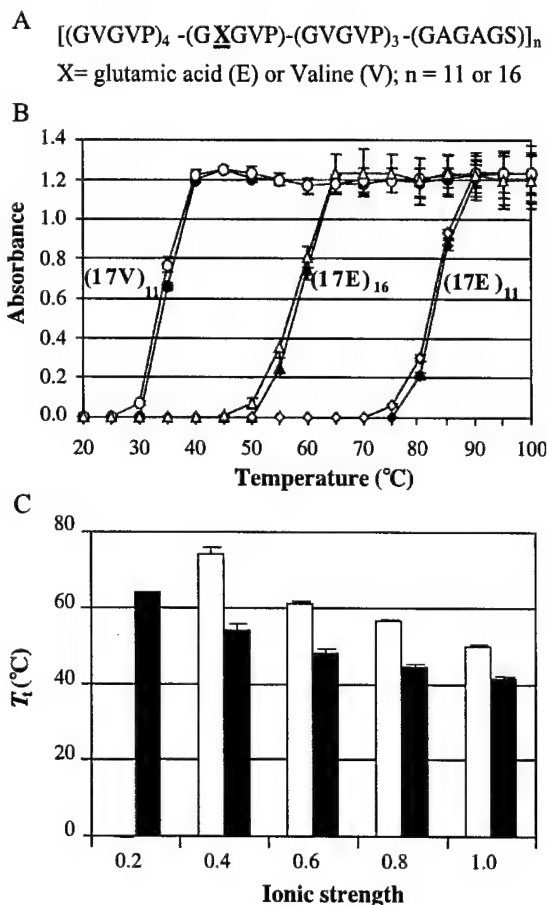


Fig. 1. (A) Structure of SELP copolymers (17E)<sub>11</sub>, (17V)<sub>11</sub>, and (17E)<sub>16</sub>. Head and tail sequences are not shown. (B) Plots of absorbance ( $\lambda = 300$  nm) of 0.5 mg/ml solutions of (17E)<sub>11</sub>, (17V)<sub>11</sub>, and (17E)<sub>16</sub> at pH 6.0 and ionic strength 0.2. Filled symbols represent heating data and open symbols represent cooling data. Each data point is a mean  $\pm$  standard deviation of triplicate readings. (C) Influence of molecular weight on the solution transition behavior of 0.5 mg/ml solutions of polymers (17E)<sub>11</sub> (open bars) and (17E)<sub>16</sub> (filled bars) at pH 7.0, and ionic strength ranging from 0.2 to 1.0. All  $T_i$  values are mean  $\pm$  standard deviation of triplicate readings. From Ref. [68].

the molecular weight of SELPs and strategic placement of ionizable amino acid residues within the SELP backbone can be utilized to control the responsiveness of these polymers to environmental stimuli. Genetic engineering techniques offer a synthetic strategy by which these variables can be controlled with a fidelity that is not achievable by chemical synthesis.



The influence of environmental conditions such as pH, temperature and ionic strength on the equilibrium swelling ratio of hydrogel forming SELP-47K containing four silk units in the repeat monomer (structure Fig. 2, Panel A) has been investigated [65]. Equilibrium swelling studies demonstrated that physically

crosslinked SELP-47K hydrogels were relatively insensitive to environmental changes in pH, temperature and ionic strength. This insensitivity was explained by the irreversible crystallization of the silk-like blocks. In contrast, increasing the polymer concentration (Fig. 2, Panel B) or gel curing time (Fig. 2, Panel C) at 37 °C resulted in lower hydrogel equilibrium swelling ratios [65]. Reducing the concentration or cure times diminishes the probability of interaction of polymer chains, which results in decreasing crosslinking density and leads to a higher degree of swelling. The results of the study provide insight into the mechanism of gel formation and the degree of hydration of SELP hydrogels, and have laid the foundations for the controlled delivery of bioactive agents from silk–elastinlike hydrogels, which will be described in Section 4.

Implants of SELP and a silk-like polymer containing fibronectin (SLPF) were biocompatible and showed little or no adverse immunogenic response in vivo. In most cases, the immune response was not chronic and was due to antibodies directed toward silk units, while no antibody responses toward elastin and fibronectin were detected [64,71]. Bioresorption studies on a series of SELPs showed that their biodegradation is a function of the silk and elastin block lengths, as well as block sequences, and can be systematically adjusted to tailor the resorption time to the application [72]. The variations in material properties within this class, as a function of structure, illustrate the capabilities of genetic engineering techniques for the synthesis of polymers with high degree of control over sensitivity to environmental stimuli, gelation kinetics, biorecognition, biodegradation and drug release. For more information on SELPs, the reader is referred to a recent article that reviewed their biosynthesis, structures, physicochemical properties and biological fate in detail [61].

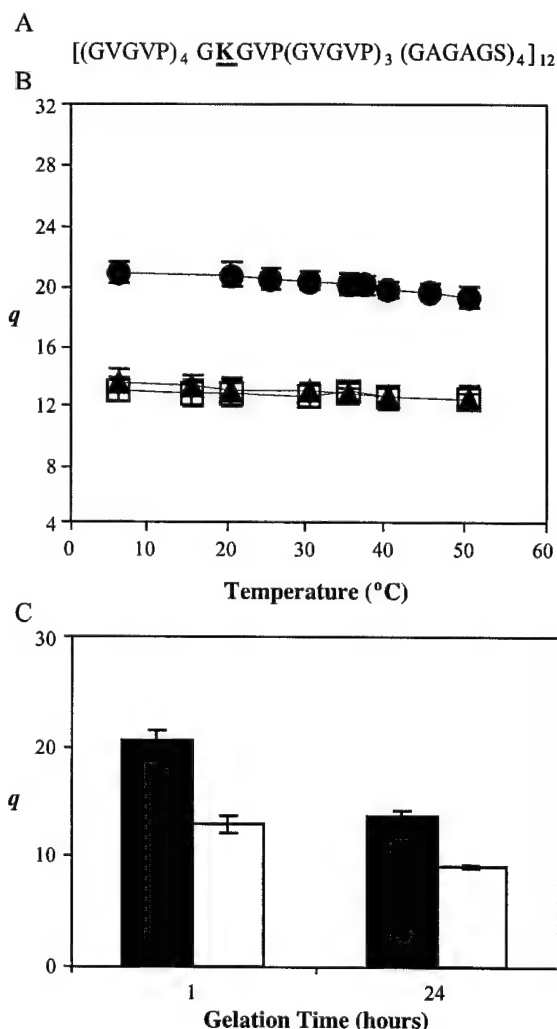


Fig. 2. (A) Structure of SELP-47K. Head and tail sequences are not shown. (B) Effect of polymer concentration on the weight equilibrium swelling ratio ( $q$ ) for SELP-47K hydrogels cured for 1 h at 37 °C in PBS (pH 7.4,  $\mu=0.15$ ), (●) 8 wt.%, (□) 10 wt.% and (▲) 12 wt.%. Symbols represent mean value  $\pm$  one standard deviation ( $n=3$ ). (C) Effect of gelation (cure) time on  $q$  for SELP-47K hydrogels stored in 1 × PBS at 20 °C (■) 8 wt.% and (□) 12 wt.%. Bars represent mean value  $\pm$  one standard deviation ( $n=3$ ). From Ref. [65].

#### 2.4. Coiled-coil and leucin-rich protein domains

Coiled-coils are left-handed super-helical bundles of more than one right-handed helix that are found in many proteins [73]. The primary structure of coiled-coils is characterized by heptad repeats of  $[(abcdefg)_x]$  with two turns covering three and a half residues. The first 'a' and fourth 'd' positions consist of hydrophobic residues (the most common are Leu, Ile and Val)

which stabilize the coiled-coil through hydrophobic interactions, while residues occupying positions 'e' and 'g' are often polar (the most common are Lys and Glu), resulting in electrostatic interaction that controls the orientation of the helices [74,75].

The structure, stability and specificity of the coiled-coil can be altered by substitution of the hydrophobic core residues or the more polar residues responsible for the inter-helical ionic interactions [76]. The substitution of the hydrophobic leucine and valine residues with more hydrophobic trifluoro-derivatives increase the stability of the coiled-coil structure [77,78] while substitution with polar residues contributes more to structural specificity and less to stability [79]. Instability of the coiled-coil structure results in unfolding of the protein, causing collapse and contraction, a useful property that may be used to trigger the collapse of polymeric matrices for controlled release. In addition, coiled coils have potential for use as generic docking devices for two-step drug targeting approaches [76].

Recombinant DNA methods have also been used to create another class of artificial protein-based polymers that undergo reversible gelation in response to changes in pH and temperature. These are triblock copolymers, described generally as rod-coil-rod, where a flexible, water-soluble polyelectrolyte segment is centered between terminal leucine-rich domains, known as "leucine zipper" [75].

The leucine-rich motif is characterized by the previously mentioned heptad periodicity, common to coiled-coil proteins, generally designated *abcdefg*, where 'a' and 'd' are hydrophobic amino acids (frequently leucine, especially at position 'd' and the residues at positions 'e' and 'g' are usually charged) [80]. Under appropriate conditions of pH and temperature, such heptads acquire  $\alpha$ -helical conformation, placing the hydrophobic 'a' and 'd' residues on the same side of the helix. Aggregation, most often in the form of coiled-coil dimers, is then promoted by inter-helical hydrophobic interaction, whereas pH-dependent interactions between the 'e' and 'g' residues modulate the stability of the coiled-coil aggregates. Aggregation number [81], dimerization specificity [82], aggregate stability [83] and aggregate structure [84] can be manipulated within wide limits through the control of the primary structure of the peptide. The central polyelectrolyte

domain [85] usually consists of a water-soluble peptide sequence lacking regular secondary structure [1], which is considered a desirable feature for the design of highly swollen hydrogels [86].

Aggregation of the terminal leucine-rich domains triggers the formation of a three-dimensional polymeric network, with the polyelectrolyte segment retaining solvent and preventing precipitation of the entire chain. Upon increasing the pH or temperature, the leucine-rich domain aggregates dissociate and the gel dissolves into a viscous polymer solution [85].

The effect of pH and temperature on gelation of protein-based polymers containing terminal leucine-rich domains was investigated by diffusion wave spectroscopy, measuring the time-averaged mean-square displacement (MSD) of tracer particles as a function of time. Increasing temperature or pH lead to deaggregation of the gels into viscous liquid, in which the tracer particles freely diffused, as demonstrated by a linear increase of MSD with time. At lower temperature (23 °C) or pH, the elasticity of the gels limited the magnitude of the tracer particle fluctuations and resulted in a plateau in the MSD [85]. While particle tracking in these gels was done to evaluate their gelation properties, the variability of particle diffusion within the three dimensional network as a function of environmental stimuli has clear implications for the diffusion of solutes within matrices for controlled drug delivery applications.

### 2.5. $\beta$ -Sheet forming polymers

Sequences consisting of alternating polar and non-polar amino acid residues form  $\beta$ -sheet structures through an anti-parallel  $\beta$ -strand pattern. A protein-based polymer that has the tendency to form  $\beta$ -sheets has been synthesized, based on the "EAK" motif, consisting of AEAEAKAK sequence repeats [87]. This sequence assumed a  $\beta$ -sheet structure even at a low concentration of 0.625  $\mu$ M. The  $\beta$ -sheets of poly EAK aggregate to form well-defined amyloid-like fibrils, approximately 10–20 nm in diameter, which form gels at neutral pH. The secondary structure of the polymer is stable under wide range of solvent conditions as well as in the presence of urea, which is known to disrupt  $\beta$ -sheets, and increasing the temperature resulted only in minor changes in the aggregation of the  $\beta$ -sheets of poly EAK [88].



## 2.6. Alanyl glycine polymers

Recombinant DNA methods have been used for successful expression of a variety of [AlaGly] repeats, also known as *poly(alanyl glycine)* or *poly(AG)*, which form  $\beta$ -sheet structures similar to those formed by silk fibroin [89–96]. Sequence modifications in these polymers revealed the importance of polymer sequence in controlling of structural organization and crystalliza-

tion. The X-ray diffraction pattern of genetically engineered poly(AG) suggests that it crystallizes in the form of crystal-folded lamellae that consist of polar  $\beta$ -sheets folding through  $\gamma$ -turns with like surfaces of either alanine or glycine stacking together [95].

McGrath et al. studied a series of genetically engineered  $[(AG)_3PEG]_n$  polymers [90]. Interestingly, these polymers contained alanyl glycine repeats which can adopt an antiparallel  $\beta$ -sheet arrangement, with

Table 1  
Some examples of genetically engineered polymers

Class	Description	General structure	Physicochemical properties	References
Pronectin F <sup>TM</sup>	Silk-like polymer with fibronectin loops	$[(GAGAGS)_9GA AVTGRGDSPASAAGY]_n$	Crystalline structure with cell attachment motif	[60]
ELP	Polymers made of elastin repeats	$[VPGXG]_n$ ( $X$ =any amino acid except proline)	Elastic chains that assemble above their phase transition temperature $T_i$	[15,23]
SELP	Block copolymers of silk and elastin units	$[(GAGAGS)_m(GVGXP)_n]_o$ ( $X$ =any amino acid except proline)	Soluble, fluid, injectable. Can form physically crosslinked hydrogels	[69,102]
Poly $[(AG)_3PEG]$	Polymer of nonapeptide repeat $(AG)_3PEG$	$[(AG)_mPEG]_n$	Amorphous glass crystals soluble in water	[89,90]
Hybrid poly (HPMA-co-DAMA)-coiled coil	Poly(HPMA-co-DAMA) crosslinked with coiled coil protein domains	Crosslink: $-[(abcdefg)_n]-(a,d: \text{hydrophobic residues})$	Conformation change to random coils upon raising temperature	[105]
Poly (CS <sub>5</sub> ELP)	Elastin-like blocks spaced by fibronectin domains	$[(GEEIQIGHIPREDVDYHLYP)(GVGXP)_m]_n$ ( $X$ =any amino acid except proline)	Thermal transition behavior and cell attachment activity	[27]
Leucine zipper-polyelectrolyte domain	Coiled coil leucine zipper flanking Ala–Gly-rich domain	$[(abcdefg)_n]-(AG)_3PEG_{110}-[(abcdefg)_n]$ ( $d$ =leucine; $e,g$ =charged residues)	pH and temperature-dependent $\alpha$ -helix with central water-soluble random coil	[85]
Spider dragline silk-like polymer	Silk-like polymer based on analogs of two consensus repeats of spider dragline silk	1. $[GGAGQGQGYGGLGSQGAGRGG LGGQGGAG]_n$ 2. $[GPGGYGGPGQGGPGGYAPGQQ PSGPGS]_n$	$\beta$ -sheet crystals, insoluble in most solvents, resist digestion by proteolytic enzyme	[42]
Chimeric SLP	Combination of two different silk-like motifs	$[(GAGXGS)_n(A)_{18}]$ where $X$ =tyrosine or valine	Some $\alpha$ -helical structure which improved solubility of silk	[55]
Poly “EAK”	Polymers of EAK amino acid repeats	$[AEAEAKAK]_n$	Amyloid-like highly stable $\beta$ -sheet fibrils	[88]
PLGA	Polymers of $\alpha$ -L-glutamic acid	$[(E)_{17}D]_n$	Rod-like polymer	[97]
Poly(AG)	Monodisperse copolymer of (L-alanyl glycine)	$[AG]_n$	Antiparallel $\beta$ -sheet silk-like polymer	[95]
Poly $(AG)_3EG$	Homopolymer of octapeptide AG rich intercepted with E residue	$[(AG)_3EG]_n$	$\beta$ -sheet silk-like structure forming needle-shaped lamellar crystals	[91]

proline residues capable of initiating  $\beta$ -turns. However, the primary structure also contained a combination of proline and glutamic acid, which restrains the  $\beta$ -sheet formation. Differential scanning calorimetry, X-ray scattering and Fourier transform infrared spectroscopy showed that the polymers formed an amorphous glass with no evidence of  $\beta$ -sheet structure. The use of an odd number of amino acids in the alanyl-glycine repeat prevented the formation of a sufficient number of hydrogen bonds required to stabilize the  $\beta$ -sheet and allowed the formation of  $\beta$ -turns at only one of the edges of the  $\beta$ -sheet [90]. The partial replacement of proline residues by the artificial amino acid azetidine-2-carboxylic acid resulted in the restoration of the  $\beta$ -sheet structure of polymer in the solid state [93].

Studies by Krejchi et al. [91] focused on another type of recombinant alanyl-glycine polymer. They designed and synthesized a series of  $[(AG)_mEG]_n$  polymers, with the objective of exploring the potential to control the crystal structure. These polymers formed needle-shaped, lamellar crystals, where the thickness could be controlled by the periodicity of the amino acid sequence. Further studies on the relationship between the volume of the amino acid residues and the dimension of polymer crystalline units of  $[(AG)_3XG]_n$  (where  $X = N, F, S, V$  or  $Y$ ) showed that spacing between chains increases with increasing volume of the substituted amino acid, while the hydrogen bond and chain directions remained invariant [94]. Genetic engineering techniques also allowed the incorporation of artificial amino acids into poly  $[(AG)_3XG]$  where  $X$  is *p*-fluorophenylalanine residue. The resulting polymer adopted an antiparallel  $\beta$ -sheet conformation in the solid state [92]. The successful incorporation of artificial amino acids broadens the range of material properties that can be attained and allows more options for the derivatization of the polymer chains that could be useful for controlled release and other biomedical applications.

### 2.7. Recombinant poly(glutamic acid) polymers

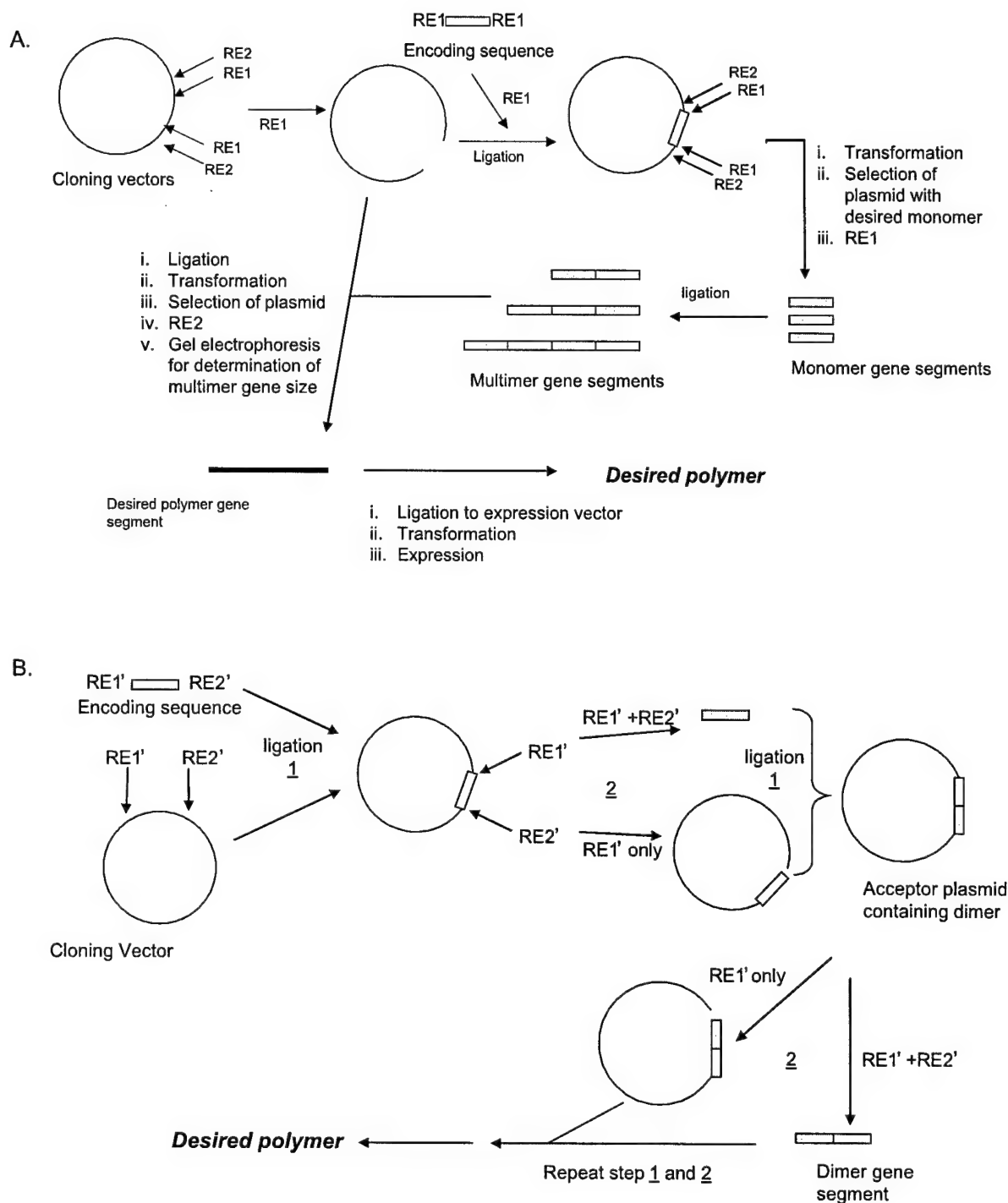
Biosynthesis of monodisperse poly(L-glutamic) acid derivatives have been reported [97–99]. The synthesis of poly(L-glutamic) acid derivatives by chemical methods results in a heterogeneous molecular weight, which complicates the evaluation of the hydrodynamic and spectroscopic properties of the

polymer. The biosynthesis of poly(L-glutamic) acid derivatives  $[(E)_{17}D]_x$  by genetic engineering techniques yielded a monodisperse, rod-like polymer with well-defined physicochemical properties [97] and lead to a series of new applications, such as preparation of poly( $\gamma$ -benzyl- $\alpha$ -L-glutamate) (PBLG) and poly( $\gamma$ -4-(hexadecyloxy)benzyl- $\alpha$ -L-glutamate) (PHBG) that show smectic ordering in solution and in films [98,99]. It is intriguing to note that recombinant techniques have been used to biosynthesize fusion proteins of a defined size and sequence composed of poly glutamic acid linked to interferon to alter the biodistribution and half-life of the therapeutic protein [100]. This was done to obviate the need for chemical modification of interferon by poly(ethylene glycol) (Table 1).

### 3. Synthesis and characterization

The biosynthetic strategy for the production of protein-based polymers involves the design and construction of synthetic genes encoding tandem repeats of a target polymer with desired sequence and length, followed by cloning and expression of these DNA sequences, using recombinant techniques. Since automated DNA synthesizers are currently limited to the production of oligonucleotides of about 100 bases, sequences encoding medium to high molecular weight polymers cannot be conveniently obtained by direct synthesis of the entire gene. Although a number of different strategies have been developed to assemble synthetic genes for such polypeptides, most methods have focused on the generation of a library of oligomeric genes by concatamerization or multimerization (Scheme 1, Panel A) of a monomer gene [2,6,15,44, 69,89,101]. Starting from a monomer gene allows the entire monomer to be synthesized chemically, or to be constructed from oligonucleotides that are accessible by chemical synthesis.

Synthesis of a monomer gene starts with the design of an oligonucleotide with the required sequence. The design of the gene should consider minimizing tandem repeats of codons, which create unstable genes subject to deletion or rearrangement. This can be accomplished by utilizing the degeneracy of the genetic code or by constructing longer monomers, encoding multiple oligopeptide blocks [102]. In addi-



Scheme 1. Two strategies for the biosynthesis of protein-based polymers: (A) concatemerization, (B) RDL.

tion, the design often includes recognition sites for restriction endonuclease (RE) enzymes flanking the portion of the gene encoding the polymer. The template and complementary oligonucleotide strands are then synthesized, annealed, purified and digested by suitable RE(s) (Scheme 1, Panel A). The purified monomer is then inserted into a cloning plasmid by utilizing the same restriction sites.

In order to make polymeric genes, the DNA monomer must be concatamerized. This can be accomplished by producing large amounts of monomer by plasmid preparations or polymerase chain reaction (PCR) and incubating the monomers in the presence of DNA ligase. Self-ligation results in a variety of different concatamer sizes. The concatamer of interest may then be isolated by gel electrophoresis and inserted into an appropriate cloning or expression vector. Alternatively, the concatamer mixture can be incubated with the vector, in the presence of DNA ligase, yielding a series of plasmids with genes encoding polymers of different molecular weights. In either case, the concatamer (or concatamer mixture) is cloned into a vector, transformed into *Escherichia coli*, and its length and sequence are verified by restriction digestion, gel electrophoresis and DNA sequencing. Once the sequence has been verified, the plasmid containing the desired polymer gene can be transformed into an expression host, which will produce the polymer. Depending on whether or not an expression plasmid was used for the cloning of the concatamer, this process may require an additional

cloning step prior to transformation (Scheme 1, Panel A) [69,102].

Concatamerization has the advantage of creating a library of multimer gene segments in a single ligation step. The process is fast and can be used with large (multimilligram) quantities of DNA. However, this

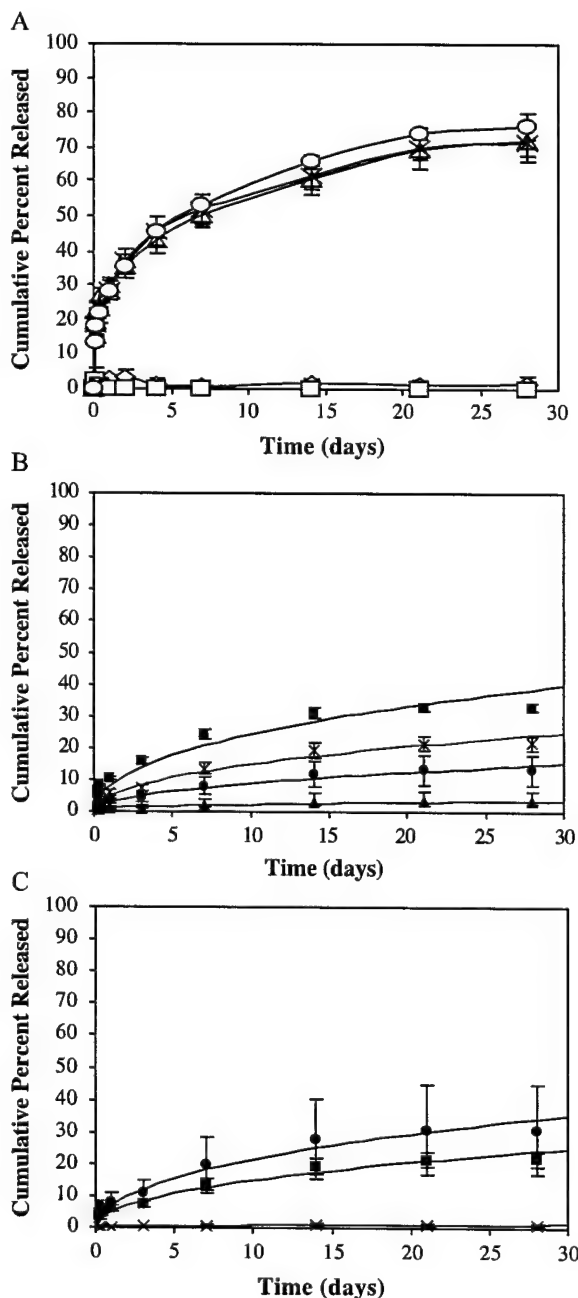


Fig. 3. (A) Cumulative release of pRL-CMV from 12% (w/w) SELP-47K hydrogels, in PBS with  $\mu=0.03$  M ( $\diamond$ ),  $0.10$  M ( $\square$ ),  $0.17$  M ( $\Delta$ ),  $0.25$  M ( $\times$ ), and  $0.50$  M ( $\circ$ ). Hydrogels were cured for 1 h at  $37^\circ\text{C}$  before placement in the appropriate buffer. Gel dimensions:  $r=2.9$  mm,  $h=1.6$  mm, prepared by molding technique. Each point represents average  $\pm$  standard deviation ( $n=3$ ). From Ref. [116]. (B) Effect of plasmid DNA size on *in vitro* release from SELP-47K hydrogels: ( $\blacksquare$ ) pUC 18 [2.6 kbp], ( $\times$ ) pRL-CMV [4.08 kbp], ( $\bullet$ ) pCFB-EGSH-Luc [8.5 kbp], ( $\blacktriangle$ ) pFB-ERV [11 kbp]; (—) theoretical release. Gel dimensions:  $r=2.3$  mm,  $h=2.8$  mm,  $v=50$   $\mu\text{l}$ , prepared by syringe technique. Each data point represents the mean  $\pm$  standard deviation for  $n=3$  samples. (C) Effect of pRL-CMV conformation on *in vitro* release from SELP-47K hydrogels: ( $\bullet$ ) linear, ( $\blacksquare$ ) supercoiled, ( $\times$ ) open-circular; (—) theoretical release. Gel dimensions:  $r=2.3$  mm,  $h=2.8$  mm,  $v=50$   $\mu\text{l}$ , prepared by syringe technique. Each data point represents the mean  $\pm$  standard deviation for  $n=3$  samples. Panels B and C are from Ref. [67].

strategy suffers from a lack of precise control over the ligation, resulting in a population of DNA oligomers with a distribution of different lengths [5]. The average degree of oligomerization can be partially controlled by varying the ligation conditions, but concatamerization does not guarantee the synthesis of a gene with a desired length. Thus, concatamerization is most useful when a range of MWs must be rapidly generated, but not for the synthesis of a gene with a specified molecular weight.

In addition to limitations on control over polymer size, the concatamerization strategy is impaired by the dependence on a limited pool of restriction enzymes that recognize nonpalindromic cleavage sites. These enzymes are used to generate DNA monomers that can undergo self-ligation to form multimers. Sometimes, unnecessary amino acid residues are introduced into the polymer sequence in order to make compatible restriction sites [6].

An alternative synthetic strategy that limits the introduction of undesired amino acid residues into the polymer sequence during concatamerization is called “seamless cloning” [6]. The significance of this approach resides in the fact that type II(s) restriction endonucleases digest DNA downstream of their

recognition sites. Cleavage of DNA with the type II restriction endonuclease *Eam*1104 I generates a 5' overhang with a sequence that is independent of the recognition site (5'-CTC TTC N<sup>^</sup>NV NNN-3'). This general procedure can yield any triplet sequence at the 5'-terminus of the gene segment, which overcomes the need for a group of specific endonucleases with unique internal recognition and cleavage patterns. Moreover, the *Eam*1104 I recognition sites are cleaved from the gene monomer segment, and thus not incorporated into the coding sequence of the DNA monomer. The seamless cloning strategy has been utilized to clone artificial genes encoding ELPs and poly(EAK) [6,87].

A third synthetic approach, called “recursive directional ligation” (RDL), was developed for the synthesis of protein-based polymers with a specified chain length and sequence (Scheme 1, Panel B) [5]. This approach involves stepwise oligomerization of DNA monomers to yield a library of oligomers ranging from the monomer to an oligomer of a required length. First, a synthetic oligonucleotide segment, encoding the desired monomer gene is ligated into a cloning vector. The oligonucleotides are designed so that two compatible cohesive ends are

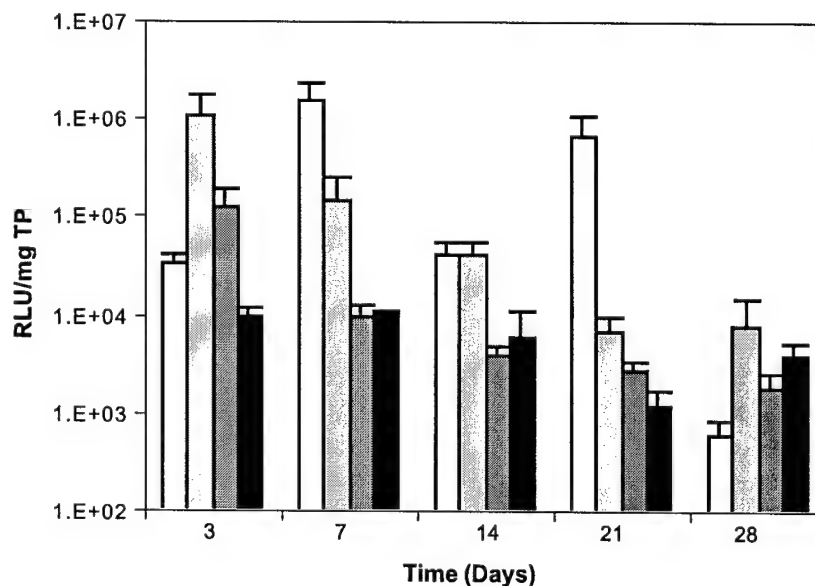


Fig. 4. Expression of *Renilla* luciferase in MDA-MD-435 tumors grown subcutaneously in athymic *nu/nu* mice, after intratumoral injection. Bars represent 4 wt.% polymer (white), 8 wt.% polymer (light gray), 12 wt.% polymer (dark gray), and naked DNA without polymer (black). Each bar represents the mean  $\pm$  standard error of the mean for  $n=4$  or  $n=5$  samples. From Ref. [67].

produced upon cleavage, using two different restriction enzymes, where the choice of the enzymes would be polymer-specific (i.e. RE1' and RE2'). This enables the annealed product to be directly incorporated into the linearized, cleaved cloning plasmid at the corresponding restriction endonuclease site. The dimerization step involves the production of an insert by cleaving the cloning plasmid, containing the monomer gene segment, with both RE1' and RE2', followed by subsequent ligation of that insert to a linearized plasmid treated only with RE1', resulting in dimerization of the gene and ligation to the vector. Subsequent rounds of RDL are similar to the first round, except that products from previous rounds serve as the source of the insert and vector. The monomer gene is designed such that the insert is ligated with its directionality preserved in a head-to-tail orientation upon ligation into the vector. The design should also ensure that the original recognition sites for RE1' and RE2' are maintained at each end of the dimerized gene, but neither recognition site is generated at the internal site of ligation. Therefore, the ligation product in any round of RDL (e.g., dimer in the first round) can be used in future rounds of RDL as the insert and/or the vector.

In order to use this synthetic technique, the restriction enzymes for RDL must satisfy four important requirements [5]: First, they must have different recognition sequences, in order to allow selective digestion either by one or by both of the enzymes, and hence both sites do not exist at the internal site of ligation. Second, the two enzymes must produce complementary, single-stranded DNA overhangs upon cleavage. Third, at least one of the two sites should be solely present in the inserted gene segment so that treatment with the enzyme cleaves the plasmid only at the insert. Finally, and most importantly, the recognition sequences of both REs must be compatible with the coding sequence of the desired polymer such that, upon ligation of the two gene segments, the polymer sequence is not disrupted at the internal site of ligation.

Several approaches have been utilized for the purification of protein-based polymers. Recombinant proteins are typically purified using a fusion tag, which may be located on either the N- or C-terminus of the protein. Some protein-based polymers can be purified by exploiting their special physicochemical

properties. For example, McPherson et al. [15] have utilized temperature-induced aggregation to purify ELPs. Interestingly, this technique has been extended to the generalized purification of other proteins, through the incorporation of terminal ELP-tags [23,103]. Biopolymers consisting of repeats of  $[(AG)_3PEG]_x$  were purified by stepwise pH changes, followed by ion-exchange chromatography [1]. Another approach involved the expression of protein-based polymers  $[G-(VPGVG)_{19}-VPGV]$  in the form of glutathione-S-transferase fusion protein that was then purified by chromatography, followed by liberation of the biopolymer by cleavage with protease factor Xa [15]. A widely used method relies on the presence of several histidine residues on the N- and/or C-terminus of the desired sequence. The expressed protein-based polymer is then purified by metal chelate binding chromatography [69,104,105].

Following purification, the protein can be characterized by conventional techniques. These frequently include amino acid content analysis, size exclusion chromatography, mass spectrometry and circular dichroism spectroscopy.

#### 4. Biomedical applications

Protein-based polymers have shown potential in a variety of biomedical applications, such as injectable urethral bulking agents for the treatment of female stress urinary incontinence [106], cell culture surfaces containing biorecognition sequences for cellular attachment [60], tissue engineering [21] and drug delivery [9]. Some of these applications are discussed below with an emphasis on controlled release.

##### 4.1. Controlled release of bioactive agents

The design and development of injectable drug delivery systems capable of forming biodegradable networks upon injection has been the focus of many studies over the past few years [107–111]. These systems generally consist of polymers capable of forming viscoelastic gels under physiological conditions, or upon exposure to stimuli such as UV light [110], temperature [107–109] or a non-solvent [111].

The synthesis of polymers using genetic engineering techniques offers the ability to control the micro-



structure, function and fate of biomaterials at the molecular level for controlled release. An additional advantage of genetically engineered protein-based polymers is that aqueous-based vehicles may be used to produce biocompatible, biodegradable and resorbable, in situ gel-forming implants, which allow delivery of a variety of bioactive agents.

Generally, the release of a solute from polymeric networks is affected by the physicochemical properties of the network and the solute, and any solute-network interactions. Genetically engineered polymers have been chemically crosslinked [22] or physically crosslinked [61] semisolid matrices for drug delivery applications. Some specific examples are discussed below.

#### 4.1.1. Drug delivery

*4.1.1.1. Controlled release from crosslinked systems.* One class of polymers studied intensively for potential as in situ gel-forming depots is the silk-elastinlike class of polymers. In clinical applications, bioactive agents can be mixed directly with aqueous SELP solutions at room temperature, prior to administration and gelation of the mixture. At body temperature, SELP solutions irreversibly self-assemble via hydrogen bond-mediated physical crosslinks, forming hydrogels [64].

Diffusion of macromolecules through SELP hydrogels has been characterized. Cappello et al. [64] studied the release of fluorescent probes with molecular weights ranging from 380 to 70,000 from SELP hydrogels with different compositions. There was a negative correlation between the molecular weight of the probes and their release rates from a gel with a certain composition. Variations in the rates of release were due to differences in the physicochemical characteristics of the molecules and the pore size of the hydrogels.

The delivery of peptides and proteins in a sustained and localized fashion has been the focus of many studies [112,113]. The release of the recombinant mitotoxin Pantarin, which inhibits the proliferation of tumor cells expressing fibroblast growth factor, was investigated from SELP-47K hydrogels [64]. In the first day, Pantarin showed a rapid initial release followed by a slow and sustained release of approximately 1% per day for at least 8 days. Although

hydrogel formation did not affect the bioactivity of the protein, the time allowed for gelation of Pantarin/SELP-47K solution affected the amount of Pantarin released in the initial phase, where larger amounts of Pantarin were released at shorter gelation times [64].

The release of three hydrophilic compounds: theophylline (MW 180), vitamin B12 (MW 1355) and cytochrome C (MW 12,384) exhibited Fickian, size-dependent diffusion behavior from 12 wt.% SELP-47K hydrogels [66]. Though size-dependent release was observed at different gelation times, an increase in gelation time from 1 to 24 h lowered the normalized diffusivity of small molecular weight theophylline and vitamin B12 but not for cytochrome C. Solute partitioning results indicated that vitamin B12 was slightly preferred by SELP-47K hydrogels as compared to theophylline and cytochrome C. These results suggest that the partitioning of solutes into SELP-47K hydrogels is not attributed to size exclusion alone, but that interaction between the solute and the polymer also plays a role. Increasing the gelation time changes the porosity, pore size distribution and tortuosity of the network, which affect the transport of the solutes. The release of theophylline was affected by the gelation time and not the partition coefficient of theophylline, which suggests that longer gelation time resulted in a more tortuous diffusion path length for small molecules. Although the gel dimensions remained essentially unchanged during release, a significant increase in the hydrogel equilibrium swelling ratio was observed at the end of these release experiments, which is most likely due to the release of the polymer soluble fraction over the course of the study.

Genetically engineered ELPs can be designed to control drug loading or release based on their inverse transition temperature [22]. Increasing the hydrophobicity of the polymer or the plastic nature of chemically crosslinked ELP matrices can be used to obtain sustained release of neutral drugs, while controlled loading and release of ionizable drugs is attainable by designing ELPs carrying ionizable functional groups [22,114].

The loading and release of several ionic probes and drugs, such as Biebrich scarlet red, Naltrexone, Dazmegrel and Leu-enkephalin, from chemically crosslinked, genetically engineered ELP-based hydrogels composed of poly(GVGVP), poly(GVGIP) and poly(AVGVP) have been studied [22]. Below  $T_i$  the matrix

swells and imbibes the drug solution. The drug is then released by raising the temperature, due to the contraction of the matrix. The diffusional release profile of Biebrich scarlet red from poly(GVGVP) hydrogels showed a sustained release pattern over a period of 11 days. The partial replacement of the valine at position 2 with asparagine resulted in faster release from the matrix. This was attributed to the transformation of the asparagine to anionic aspartic acid, inducing swelling and disruption of the matrix [22]. On the other hand, the ionizable drugs, Naltrexone, Dazmegrel and Leu-enkephalin showed only a limited sustained release, apparently due to the absence of ionizable functional groups in the ELP structure that could potentially control the release by ion-pairing.

Loading ELP matrices with hydrophobic drugs, norgestrel and dexamethasone was also studied [22]. Matrices composed of poly(GVGVP), poly(GVGIP) and poly(AVGVP) were loaded with 40%, 28% and 11% by weight dexamethasone and 10%, 43% and 8% by weight norgestrel. These high levels of loading of hydrophobic drugs were achieved by lyophilizing swollen matrices followed by mixing the hydrophobic drug with the lyophilized matrix in 90% alcohol.

*4.1.1.2. Controlled release from non-crosslinked systems.* Urry et al. [22] investigated the transductional loading and release of ionic drugs from ion-pair loaded coacervates. Loading of ionizable drugs, such as the cationic drugs Naltrexone and Leu-enkephalin on an ELP containing glutamic acid residues, poly(GFGVP GEGVP GFGVP), resulted in lowering the  $T_i$  of the polymer [22]. This reduction in  $T_i$  was a function of the position of glutamic acid residue in the monomer sequence. Similarly, loading of anionic drugs on an ELP containing cationic amino acid residues such as poly [0.69 (GVGIP), 0.31 (GKGIP)] resulted in lowering the  $T_i$  of the polymer [22].

The effect of changing the  $pK_a$  of the ELP on the release profile of Naltrexone was studied by synthesizing glutamic acid-containing polymers with different degrees of valine substitution with the hydrophobic residue phenylalanine [22]. Increasing the hydrophobicity of the ELP is accompanied by an increase in the energy of the charged state, and hence an increase in the  $pK_a$  shift of the carboxyl moieties and affinity for an oppositely charged species [22]. Results showed that the release profile of the drug

from the coacervate is a function of the hydrophobicity of the polymer [22], where more hydrophobic polymers, showing a greater  $pK_a$  shift exhibited sustained release over a longer period of time. Similar studies on a pair of ELPs that differed only in the type of anionic residue, one with glutamic acid and one with aspartic acid, showed that the polymer with the larger hydrophobic-induced  $pK_a$  shift exhibits a more sustained release profile [22].

The release profile of Leu-enkephalin amide from two glutamic acid-containing ELPs, namely [GEGVP GVGVP GVGFP GFGFP (GVGVP)<sub>2</sub>]<sub>n</sub> and [GFGFP GEGFP GFGFP]<sub>m</sub>, which differ in the number of phenylalanine residues and glutamic acid residues per 30-mer showed that the density of the drug loaded is determined by the number of negatively charged carboxylate functional groups [114]. ELPs with a lower Glu/Phe ratio showed a lesser amount of release, due to a small shift in  $pK_a$ . However, this level of release was constant for more than 90 days, due to a greater number of glutamic acid residues, while ELPs with lower phenylalanine content showed a greater level of release, but only for 45 days. These studies suggest that the magnitude of release of ionic drug from ELP depends the hydrophobicity of the polymer while the duration of release depends on the density of ionic residues.

While some of the previously described ELPs were chemically synthesized sequential polypeptides and others genetically engineered, all utilized naturally occurring amino acids. The loading and release of drugs from these ELPs demonstrates the utility of the range of natural amino acids that can be incorporated into genetically engineered polymers for controlled drug delivery. The polymers can be tailor-made depending on the physicochemical properties of the drug and the environmental conditions under which release is desired.

The design of genetically engineered protein-based polymers for use as drug carriers is partially dictated by the method of incorporation of the drug into the carrier (e.g., conjugation, chelation, encapsulation) and the method for targeting of the drug-polymer system in vivo. Thermal targeting of recombinant ELPs has been achieved by localized hyperthermia of the target tissue [18,20]. This technique takes advantage of the ELP  $T_i$  to localize the polymer to a specific site within the body, such as a tumor. The

ELP was designed to have a  $T_i$  intermediate between body temperature ( $T_b$ ) and a higher temperature ( $T_h$ ) that was induced by localized hyperthermia, allowing localized precipitation at the heated region. Genetically engineered ELP-rhodamine conjugates, in combination with localized hyperthermia, showed a two-fold greater accumulation in heated tumors when compared with unheated tumors. In vivo fluorescence video microscopy showed that the preferential accumulation of the polymer in the heated tumor is due to increased accumulation in the vasculature and increased extravasation [18].

The thermal properties and in vitro cytotoxicity of ELP conjugated to the chemotherapeutic agent doxorubicin through an acid-labile hydrazone bond were evaluated [115]. Conjugation of doxorubicin to the polymer decreased the  $T_i$  by  $\sim 10^\circ\text{C}$ . Human squamous carcinoma cell line FaDu treated with free drug accumulated the drug primarily in the nucleus. When treated with polymer-drug conjugates, the drug was dispersed throughout the cytoplasm. The cytotoxicity of the conjugate was comparable to that of free doxorubicin. These studies indicate the potential of thermally responsive genetically engineered polymers for controlled drug delivery where by varying the amino acid sequence and length of the polymer and attachment of drugs it is possible to control thermal sensitivity and release properties.

#### 4.1.2. Controlled gene delivery

Hydrogel-forming genetically engineered silk-elastinlike polymers have been investigated as matrices for controlled gene delivery [67,116,117]. Megeed et al. [116] studied the effects of ionic strength, hydrogel cure time, DNA concentration and polymer concentration on the release of plasmid DNA from SELP-47K hydrogels. Results showed that increasing the ionic strength and decreasing both the hydrogel cure time and polymer concentration increased the rate of release, while varying the concentration of plasmid DNA between 50 and 250  $\mu\text{g/ml}$  did not influence the rate of release. The cumulative amount of plasmid DNA released from the gels increased with an increase in the ionic strength of the medium in an "on/off" fashion (Fig. 3, Panel A). This was attributed to the presence of one cationic lysine residue in the SELP-47K monomer unit that participated in an ionic interaction between DNA and the hydrogel network.

This demonstrates the potentially significant influence of relatively small changes in polymer sequence on DNA release. "On/off" release from thermally responsive, chemically synthesized hydrogels has been a subject of intriguing research [118–120]. It is conceivable that further alterations to the structure of recombinant polymers could lead to on/off release in response to changes in environmental stimuli other than changes in ionic strength.

In addition to polymer structure, polymer concentration and hydrogel cure time, it has been shown that the size of plasmid DNA, its conformation and hydrogel geometry influence the rate of plasmid release from SELP-47K matrices [67]. The release of a series of plasmids with different sizes was studied. The smallest plasmid, pUC18 (2.6 kbp) was released fastest, followed by pRL-CMV (4.08 kbp), pCFB-EGSH-luc (8.5 kbp) and the largest plasmid pFB-ERV (11 kbp), which showed less than 5% release over 28 days (Fig. 3, Panel B). The size-dependent release of plasmid DNA can be explained by the hindered diffusion of larger plasmids through the pores that are formed by physical crosslinking of SELP-47K.

The influence of plasmid conformation on release from SELP-47K hydrogels was evaluated by producing plasmid DNA predominantly in the supercoiled, open-circular and linear conformations. The cumulative release of the three conformations was in the order of linear > supercoiled > open circular (Fig. 3, Panel C) [67]. The greater diffusivity of the linear and supercoiled plasmids relative to the open-circular plasmid arises from the different topology of the three conformations. Transport of linearized DNA by reptation would be expected to increase its rate of release from SELP-47K hydrogels, as the pore size would be less restrictive to a semi-flexible, linearized molecule [121]. Supercoiled DNA, on the other hand, is known to have a larger diameter and shorter contour length than linear DNA [122]. The larger diameter of supercoiled DNA could increase its contact with the hydrogel network, leading to a rate of release that is less than that of the linearized plasmid. The essential lack of release of the open-circular form of the plasmid may be due to its impalement on the fibers that constitute the hydrogel matrix [123].

The effect of hydrogel geometry on the release of plasmid DNA was investigated by comparing release

from disc-like hydrogels ( $r=4.3$  mm,  $h=2.0$  mm) and cylindrical hydrogels ( $r=2.3$  mm,  $h=5.1$  mm) [67]. The difference in the rate of release was attributed to the difference in the surface-to-volume ratio of the two geometries and was accurately described by a mathematical model for two-dimensional diffusion from a cylindrical object. These results may be useful in determining where and how to inject SELPs for localized gene delivery.

The integrity and in vitro bioactivity of plasmid DNA encapsulated in SELP-47K hydrogels was evaluated over 28 days [67]. In vitro release studies showed that plasmid DNA remains structurally intact with regard to molecular weight and conformation. Moreover, the in vitro bioactivity of the DNA purified from the hydrogels after incubation in PBS at 37 °C for 28 days was comparable to the control DNA used to prepare the hydrogels [67].

In vivo delivery of plasmid DNA from SELP-47K hydrogels was evaluated by delivering pRL-CMV, containing the *Renilla* luciferase gene, intratumorally

in a murine model of human breast cancer [67]. Tumor transfection was significantly enhanced for up to 21 days when compared to the delivery of naked pRL-CMV (Fig. 4). The level of transfection was dependent upon the composition of the hydrogel, where 4 and 8 wt.% polymer hydrogels resulted in greater transfection than 12 wt.% hydrogels [67].

Recent efforts have explored the localized matrix-mediated delivery of viral vectors [124,125]. Controlled delivery of adenoviral particles using polymeric biomaterials takes advantage of the greater transfection efficiency provided by the adenovirus than naked DNA and control over release provided by polymers. The in vitro release and bioactivity of adenovirus containing the green fluorescent protein (gfp) gene, as a marker of gene transfer, from SELP 47K showed a clear qualitative relationship between the percentage of polymer in the hydrogel and the transfection of an HEK-293 cell line (Fig. 5), indicating that the release of adenoviruses can be modulated by changing the composition of the matrix

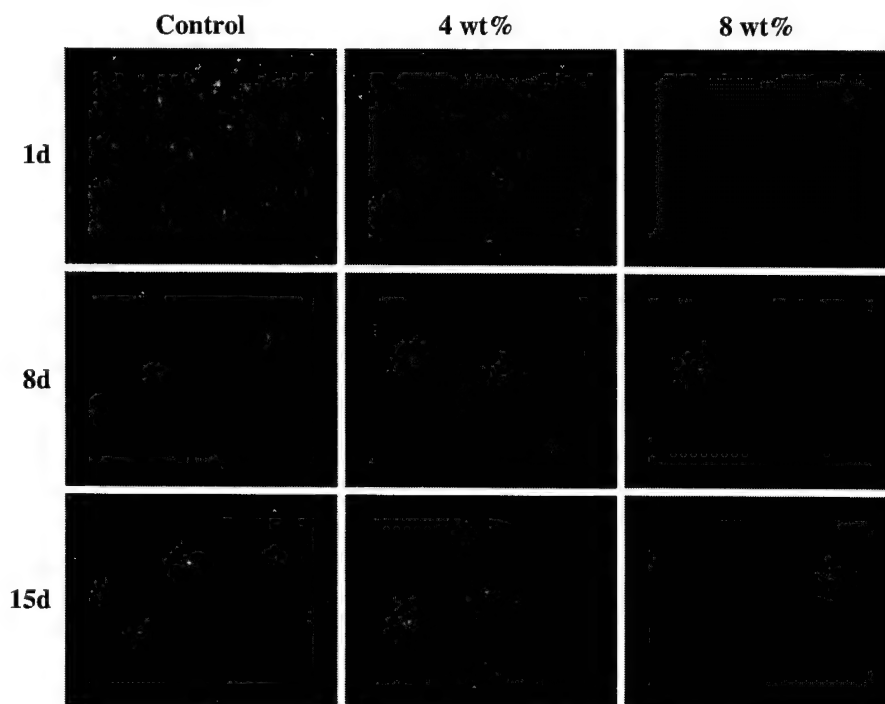


Fig. 5. Adenovirus release from SELP-47K and the corresponding bioactivity results. The percentage of polymer increases from left to right. The time of release of virus from gels used to transfect cells or control increases from top to bottom. The images are from fluorescent microscopy at 40 $\times$  magnification. Bright spots represent individual cells transfected with AdGFP. Adapted from Ref. [67].

[67]. Hydrogels composed of 4 wt.% polymer showed more release of bioactive adenovirus than 8 and 11.3 wt.% hydrogels. This is probably due to the fact that the pore sizes of the 8 and 11.3 wt.% hydrogels significantly restrict diffusion of the adenovirus in the matrix.

The simplicity of loading plasmid DNA or viral vectors into SELP hydrogels by mixing, the mild condition under which the gel formation can be controlled (absence of organic solvents and extreme pH, etc.), and the ability of SELP hydrogels to deliver plasmid DNA and adenoviral vectors *in situ* after injection through a needle suggest that these materials have potential in applications requiring controlled release of DNA or viral vectors. Coupling these properties with the ability to tailor-make structure (and therefore properties such as gelation, pore size, biodegradation, biorecognition, biocompatibility, stimuli-sensitivity, etc.) using recombinant techniques provide exciting possibilities for the design of future generations of these polymers for specific gene delivery applications.

In addition to the localized delivery of naked DNA and adenoviral particles, genetic engineering techniques have the potential to produce polymers for systemic gene delivery. A chemically derivatized, genetically engineered SLPF, namely Pronectin F, has been studied as a nonviral vector for gene delivery [126]. The cationic molecules ethylenediamine, spermidine and spermine were conjugated to Pronectin F via the hydroxyl group of serine residues, yielding Pronectin F<sup>+</sup>. When complexed with plasmid DNA at protein: DNA weight ratio of 50, complexes exhibited a zeta potential of about 10 mV and a size of approximately 200 nm. Both the zeta potential and the size of the complexes were dependent on the amount of Pronectin F<sup>+</sup> added to the plasmid DNA. *In vitro* transfection assays showed that cells treated by Pronectin F<sup>+</sup>/DNA complexes exhibited significantly greater transfection in comparison to naked DNA. The spermine derivative exhibited a transfection efficiency that was greater than the ethylenediamine and spermine derivatives. This was attributed to the higher buffering capacity of the spermine derivative, which was comparable to polyethylenimine. When compared to derivatized gelatin, containing one RGD motif, cellular attachment was greater on surfaces coated with Pronectin

F<sup>+</sup> derivatives, containing 13 RGD motifs. Fluorescently labeled plasmid showed that the uptake of plasmid DNA/Pronectin F<sup>+</sup> complexes was greater than that mediated by cationized gelatin [126]. This study shows the potential of biorecognizable genetically engineered polymers as systemic nonviral vectors for gene delivery.

The influence of polymer structure on the physicochemical properties, and hence transfection efficiency of complexes of cationic polymers and DNA is poorly understood. Studies that have sought to systematically define these relationships are limited by the inherent limitations of chemical synthesis of polymers and derivatization, such as random sequences, polydispersity and fractured structures [127,128]. The biological synthesis of cationic polymers could provide a series of macromolecules with well-defined sequences, compositions and molecular weights for DNA complexation and transfection experiments. However, limitations in cloning and expression of positively charged proteins must be overcome before the successful evaluation of structure/transfection relationships of these polymers can be studied [117]. Some initial attempts in our laboratory to clone multimer genes encoding such polymers have been described [117]. The potential of genetically engineered polymers for localized and systemic gene delivery has been discussed in detail elsewhere [117].

#### 4.2. Tissue culture and tissue engineering

Successful surgical reconstruction of damaged small- and medium-diameter blood vessels is difficult to achieve, due to thrombosis or graft occlusion, or because of neointimal hyperplasia at the junction with surrounding tissues [129]. An alternative technique based on artificial extracellular matrix proteins composed of a repeating unit structure [GVPGI]<sub>x</sub> and a cell-binding domain (designated CS5) derived from the natural extracellular matrix protein fibronectin was suggested as a vascular graft material in which the inner lining carries sequences for endothelial cell attachment [27]. The CS5 region of fibronectin was selected due to the presence of the REDV sequence that allows specific attachment and spreading of endothelial cells, but not smooth muscle cells or platelets, on artificial surfaces [130,131].

Polymers with the structures [CS5(GVPGI)<sub>40</sub>]<sub>3</sub> and [CS5(GVPGI)<sub>20</sub>]<sub>5</sub> were synthesized and evaluated for

their ability to induce attachment and spreading of human umbilical vein endothelial cells (HUVEC). Cells readily attached to substrates coated with [CS5(GVPGI)<sub>20</sub>]<sub>5</sub>, while significantly fewer cells attached to surfaces coated with [CS5(GVPGI)<sub>40</sub>]<sub>3</sub>. This may be due to the higher areal density of CS5 ligands in the former. Spreading of cells was enhanced on surfaces coated with [CS5(GVPGI)<sub>20</sub>]<sub>5</sub>, when compared to surfaces coated with fibronectin [27].

#### 4.3. Design of epitope display systems using coiled-coil motifs

Targetable polymeric drug delivery systems based on hydrophilic polymers such as *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers have been well-established as an approach for improving cancer therapy [132,133]. In these constructs, targeting moieties such as carbohydrates and antibodies have been attached to the polymeric backbone to enhance biorecognition of the delivery system. In addition, receptor-binding epitopes can be used as biorecognition moieties to target cells. Enhanced binding, due to the multivalency effect, can be achieved by incorporating multiple epitopes into a single macromolecule [134].

Coiled-coil stem loop (CCSL) peptides have been used to display receptor-binding peptide epitope sequences [75]. The coiled-coil with terminal histamine tag is attached to a solid substrate by ionic interactions, forming a rigid, brush-like structure with the loop regions exposed to the surface. When the loop region is engineered to include biorecognizable oligopeptide sequences, the epitope is presented to potential receptors or receptor-containing cells for biorecognition studies. This technique has been used to identify epitopes that are recognizable by immunocompetent cells such as CD21+ Burkitt's lymphoma cells [75,135].

### 5. Other applications

#### 5.1. Assembly of nanoparticles with recombinant block copolymers

Elastin-like blocks have been used to synthesize diblock (AB) [136] and triblock (BAB) [137] copoly-

mers, where A is a tetrapeptide or pentapeptide elastin sequence with elevated  $T_i$  values and B is [(V/I)PXYG] [138]. The sequence of the hydrophobic block (B) was chosen such that the  $T_i$  of the block occurred below 37 °C. Collapse of the hydrophobic block resulted in the formation of nanoparticles under physiologically relevant conditions. Modification of the fourth residue (Y) changed the  $T_i$  of the polypeptide in aqueous solution in a manner commensurate with the effect of the polarity of the amino acid side chains on the polymer-solvent interaction [24,70]. In addition, substitution of an Ala residue for the consensus Gly residue in the third (X) position of the repeats resulted in switching the mechanical response of the material from elastomeric to plastic [138]. These ELP domains can also be fused to molecules other than elastin, therefore, allowing the assembly of micellar aggregates that can contain structurally distinct hydrophilic sequences within the corona of the nanoparticles. In addition, the opportunity exists for the introduction of biorecognition or targeting peptide sequences (or other ligands) into the hydrophilic block, thus enabling selective targeting of nanoparticles to specific sites via receptors at the cell surface [136–138].

#### 5.2. Hybrid polymers

Hybridization of genetically engineered polymers with chemically synthesized polymers has been used to produce environmentally sensitive hydrogels [105,139,140]. These hybrids take advantage of the biocompatibility of well-established synthetic polymers and structure properties of well-defined protein motifs. Coiled-coils derived from the protein kinesin were used to crosslink a water-soluble copolymer of HPMA, resulting in two-component temperature-sensitive hybrid hydrogels [139]. The hydrogels underwent a temperature-dependent decrease in volume, due to collapse of the rod-like helical protein. Coiled-coil conformational transitions can also result from minor alterations in the primary structure or variation in pH, ionic strength and solvent composition [105]. The attachment of protein crosslinks to side-chains on a synthetic polymer backbone was accomplished through  $\text{Ni}^{2+}$  complexation of poly(histidine) fusion tags on the coiled-coil proteins.



Temperature-sensitive hydrogels of genetically engineered ELP were obtained by non-specific radical and chemical crosslinking using tris-succinimidyl aminotriacetate [141–144]. The increase in lysine content, MW and concentration of ELP of a chemically crosslinked hydrogel of ELP of structure [VPGXG] (where X=VALINE or occasional lysine every 7 or 17 pentad repeat), resulted in an increase in the total number of intermolecular crosslinks and enhanced the formation of tighter networks [144].

Halstenberg et al. used genetic engineering and photopolymerization techniques to synthesize multi-functional protein-graft-poly(ethylene glycol) polymers for tissue repair [145]. The design of the polymer included an RGD motif to enable integrin-mediated cellular attachment and protease-mediated biodegradation motifs to allow cellular infiltration of the matrix during wound healing. Hydrogels were prepared by grafting functionalized PEG to cysteine residues in the linear polymer. In vitro studies showed RGD-mediated cellular attachment and three-dimensional cellular migration that was mediated by serine protease degradation of motifs designed for this purpose [145].

### 5.3. Thin film coatings

The mechanical properties of SLPs and cellular attachment of fibronectin have been utilized for the development of thin film coatings for silicon-based central nervous system implants [146]. SLPF, a silk-like polymer containing fibronectin, showed successful adherence to both silicon substrates and target tissues, acting as an intermediate between the interface of soft tissue and the stiff silicon device [146]. SLPF has also been used to enhance the adhesion and spreading of cells on poly(styrene) culture dishes [61,71,147].

### 5.4. Heavy metal binding

The reversible phase transition properties of ELP were used to develop tunable, metal-binding biopolymers [148]. Polymers consisting of 38–78 repeating (VPGVG) blocks and one N-terminal hexahistidine were synthesized. The polymers exhibited concentration-dependent metal-binding capability when incubated with excess cadmium  $\text{Cd}^{2+}$  in a 1:1 ratio, an

observation consistent with the binding affinity of the hexahistidine tag [149]. To demonstrate the possibility of tuning the metal-binding capability of the polymers, an analog of 78 (VPGVG) with two hexahistidines at both N- and C-termini was synthesized. The metal-binding capacity of this polymer increased to a ratio of 1.5:1 [148]. The metal-binding specificity of the polymers can be tailored by employing the appropriate metal-binding domain.

As with all ELP-based polymers, increasing the salt concentration can be used to induce aggregation and precipitation at a constant temperature (isothermal precipitation). Metal removal was equally effective under isothermal and isotonic conditions [148].

## 6. Conclusions and future prospects

Control over structure at the molecular level and the diversity of motifs present in nature that can be biosynthetically combined to produce new polymers, have created interest in the genetic engineering of biomaterials for drug delivery, tissue engineering and other biomedical applications. The design and construction of ELPs, SLPs, SELPs and hybrid biomaterials are examples where such polymers have been engineered to optimize drug loading, stimuli-sensitivity, drug release, biodegradation and biorecognition. Modulation of the properties of these polymers is achieved through precise changes in sequence and length. While some examples of the influence of sequence on drug loading, aggregation, thermal sensitivity, gelation properties and DNA release and transfection were reviewed in this article, the field is still in its embryonic stages and the full potential of this synthetic strategy for controlled drug delivery has yet to materialize. Biosynthesis of protein-based polymers can allow the introduction of amino acid sequences with defined three-dimensional structures in drug delivery systems that are responsive to the physiological environment in a manner similar to the interaction of proteins with other proteins, enzymes and receptors in the body. For example the incorporation of specific molecular recognition sites that can enable receptor-mediated targeting, or catalytic sites that allow release in response to enzymatic stimuli at the target micro-environment can significantly expand the utility of these constructs in drug delivery. In addition, genetic

engineering techniques can allow the construction of a series of polymeric analogs with incremental changes in structure or molecular weight. This can be a powerful tool to elucidate the influence of polymer structure on physicochemical properties and controlled release at the organ, tissue, cellular and sub-cellular levels. These approaches can lead to the development of future generations of smart drug delivery systems that trigger release in a complex biological environment.

#### Amino acids

A/Ala	Alanine
D/Asp	Aspartic acid
E/Glu	Glutamic acid
F/Phe	Phenyl alanine
G/Gly	Glycine
I/Ile	Isoleucine
K/Lys	Lysine
L/Leu	Leucine
N/Asn	Asparagine
P/Pro	Proline
Q/Gln	Glutamine
R/Arg	Arginine
S/Ser	Serine
V/Val	Valine
Y/Tyr	Tyrosine
AB	Diblock copolymers
BAB	Triblock copolymers
CCSL	Coiled-coil stem loop
Cd <sup>2+</sup>	Cadmium
CS5	Cell binding domain
DAMA	<i>N</i> -( <i>N</i> ', <i>N</i> '-dicarboxymethylaminopropyl) methacrylamide

#### Deoxyribonucleic acid bases

C	Cytosine
N	Purine or pyrimidine bases
T	Thymine
DLS	Dynamic light scattering
ELP	Elastin-like polymers
FaDu	Human squamous carcinoma cell line
gfp	Green fluorescent protein
HPMA	<i>N</i> -(2-hydroxypropyl)methacrylamide
HUVEC	Human umbilical vein endothelial cells
MSD	Mean square displacement
MW	Molecular weight
NCMAG	<i>N. clavipes</i> major ampullate gland
NMR	Nuclear magnetic resonance

PBLG	Poly( $\gamma$ -benzyl- $\alpha$ -L-glutamate)
PBS	Phosphate buffer saline
PCR	Polymerase chain reactions
PEG	Poly(ethylene glycol)
PHBG	Poly( $\gamma$ -4-(hexadecyloxy)benzyl- $\alpha$ -L-glutamate)
RDL	Recursive directional ligation
RE	Restriction endonuclease enzymes
{[S] <sub>m</sub> [E] <sub>n</sub> } <sub>o</sub>	S is silk-like block with <i>m</i> repeats, E is elastin-like block with <i>n</i> repeats, <i>o</i> total repeats of silk-elastinlike blocks
SELP	Silk-elastin-like polymers
SLP	Silk-like polymers
SLPF	Silk-like polymers containing fibronectin
T <sub>i</sub>	Transition temperature

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## **Recombinant Polymers for Cancer Gene Therapy**

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**Brief Summary of Article:**

A major challenge for successful cancer gene therapy is the development of safe and effective gene delivery vectors. Gene delivery vectors can be viral or nonviral. Among nonviral vectors various polymeric vectors have shown potential in gene delivery. However, much work needs to be done in order to correlate polymer structure with gene release at the target site and transfection efficiency. This article is a brief introduction into cancer gene therapy, barriers and methods for gene transfer with emphasis on polymeric systems and potential of recombinant techniques for the development of novel biomaterials with defined structures for gene delivery.

## **Introduction to cancer gene therapy**

Despite advancements and new innovations in oncology, cancer continues to be a major cause of suffering and death. The main method of treatment for most solid tumors is still surgical extirpation. It is occasionally combined with radiation and chemotherapy to reduce incidence of tumor recurrence and obtain optimum local disease control. Although radiation appears to have a preferential effect on rapidly dividing cells, it is not specific in its action since it is capable of destroying both normal and malignant cells. Chemotherapeutic agents are cytotoxic drugs used often in combination to obtain a synergetic effect. However, they suffer from significant adverse reactions involving many organ systems.

The fact that cancer originates from a progression of genetic mutations, where one cell proliferates abnormally to a malignant cell population, has created great interest in applying gene therapy techniques to its treatment. During the past 15 years, approximately 585 clinical trials for treatment of human diseases using gene therapy have been evaluated (Table 1); more than 68 % of these studies are in the area of cancer gene therapy [1]. Many cancer gene therapy strategies focus on transferring genes that either stimulate or increase a specific anti-tumor immune response [2]. These strategies include methods such as the *ex vivo* transfection of T-lymphocytes to tumors and the localized delivery of cytokines that mediate inflammation and an immune response at the tumor site. Gene transfer was also used to efficiently deliver immune modulating genes into irradiated tumor cells capable of generating a biologically relevant immune response directed toward specific tumor antigens [3].

Many types of cancers evolve as a result of loss or mutation in tumor suppressor genes which act as negative regulatory genes that can overcome the uncontrolled cell proliferation driven by the activated oncogenes or other stimulatory factors. The transduction of these tumor suppressor genes may result in dormancy of the tumor cells or cell death also known as “apoptosis” [4]. An example of such a gene is p53, which has been studied extensively in cancer gene therapy [5]. Viral vectors have also been used for inhibition of the proliferation of tumor cells due to their ability to replicate and lyse tumor cells as part of their life cycle [6]. The inhibition of oncogenes by transduction of antisense oligonucleotides blocking DNA or mRNA is another growing cancer gene therapy approach.

Chemotherapy and gene therapy can be combined via suicide gene therapy where a gene introduced into a tumor converts a systemically non toxic prodrug into a cytotoxic metabolite which not only kills the transduced but also destroys the surrounding cells in a process known as the bystander effect [7]. The most commonly investigated suicide genes are the herpes simplex virus thymidine kinase (HSV-tk) and cytosine deaminase (CD) [3]. HSV-tk gene render mammalian cells sensitive to the nucleoside analog ganciclovir GCV converted to a phosphorylated compound that inhibits DNA synthesis [8]. The transduction of CD gene results in the intracellular conversion of 5-fluorocytosine into the cytotoxic metabolite 5-fluorouracil, which interferes with DNA and RNA synthesis [9]. Another evolving technique that inhibits tumor growth is the delivery of antiangiogenic genes capable of reducing the tumor vascularization and reducing its proliferation [10, 11].

## **Barriers to gene transfer**

Circumventing the biological barriers to gene delivery is a considerable challenge. Effective cancer gene therapy requires the successful transport of the transgene from the site of administration to the nucleus of the target cells where it becomes available to the transcription machinery or capable of blocking the expression of the defected genes. The barriers facing each vector may vary depending on the route of administration (Scheme 1). Biodistribution barriers for systemic delivery include the interaction with blood components and nonspecific uptake. Once in blood stream, transgenes are exposed to serum inactivation and degradation by nucleases. Thus, vectors should be capable of protecting DNA and render it inaccessible to these degradative enzymes. Protection could be achieved by encapsulation in protein capsules such as viral vectors, by condensation in polycationic lipid or polymers or by trapping in controlled release hydrogels. Whereas nonspecific uptake can be reduced by attaching targeting ligands such as antibodies [12] and peptides [13] to the surface of the DNA delivery vector.

At the cell surface the rate of entry into cells varies with cell type and occurs relatively slowly. After cellular uptake of the gene delivery systems by endocytosis, the endosomal release is another critical barrier which affects the efficiency of gene transfer since most DNA is retained in the endosomes and eventually degraded or inactivated by lysosomal enzymes. A number of strategies have been developed to enhance endosomal release. One involves using fusogenic peptides or lipids to disrupt the endosomal membrane [14]. Another strategy depends on using gene delivery systems with high buffer capacity known as “proton sponge” presumably able to reduce the acidification of the endosome and result in swelling and rupture of the endosome [15].



Following the release from the endosome, the transgene traffics toward the nucleus through the cytoplasm where it can be exposed to degradation by cytosolic nucleases. The transfection efficiency of the DNA may also be reduced by the inability to dissociate from the delivery vector as only free DNA can be imported into the nucleus. Whether the nuclear uptake depends on active transport or diffusion or a combination of both mechanisms is not clearly understood. However, the incorporation of short peptide sequences known as “nuclear localization signals” into delivery vectors has shown to enhance DNA delivery to the nucleus via active transport along microtubules [16]. Following nuclear uptake, the transgene is transcribed into messenger RNA (mRNA), which is exported to the cytoplasm and translated to the desired protein.

In addition to those barriers, other biological barriers may challenge the efficiency of cancer gene therapy. For example, crossing the vascular endothelium and migration through the interstitium to reach the tumor cells are also important barriers for the delivery of both systemically and intratumorally administered vectors to solid tumors.

### **Methods of gene transfection**

Naked DNA has been used successfully when locally injected into the tumor [17] or as DNA vaccines [18], however it is highly prone to tissue clearance and totally inefficient for systematic delivery [19]. Vectors for gene delivery can be divided into two major categories: i) viral vectors such as retrovirus, adenovirus, adeno-associated virus, herpes simplex virus, and vaccinia virus and ii) nonviral methods such as naked DNA, gene gun, liposomes, polymers, peptides and combinations thereof [20].

Viruses have evolved mechanisms of overcoming the most significant biological barriers. Therefore replication-defective viruses with viral coding sequence partially or completely replaced by therapeutic genes have been the focus of many basic research and clinical studies. Although viral vectors are highly efficient in gene delivery they suffer from several drawbacks such as limited DNA loading capacity, toxicity, immunogenicity and potential replication of competent viruses.

The limitations of viral vectors make synthetic vectors an attractive alternative. Non-viral vectors are less toxic and immunogenic and simple to produce on a large scale. Their efficiency, however, is less than that of their viral counterparts. In addition, physical properties such as size and charge play a critical role in their efficiency. In order to increase the transfection efficiency, non-viral delivery systems have been engineered to mimic the viral delivery systems by incorporation of condensing or encapsulation agent to protect DNA from nucleases, targeting ligands to reduce nonspecific uptake, endosomal release agents to avoid lysosomal degradation and nuclear localization signals to enhance nuclear uptake.

### **Recombinant polymers**

Majority of polymers used in drug and gene delivery are synthesized by chemical methods or obtained from natural sources. Chemical synthesis generally produces random copolymers with unspecified monomer sequences and statistical distributions of molecular weights and monomer compositions. This heterogeneity can yield inconsistency in the biological fate and mechanical properties of such polymers. Moreover, chemically synthesized polymers may contain residual organic solvents,

which adversely affect DNA and/or viral vectors. Incomplete reactions produce final products with trace amounts of monomer residues which can result in toxicity. Alternatively, naturally-derived polymers, such as collagen matrices used in adenoviral delivery, are limited by batch to batch variability, limited control over crosslinking density and therefore release, and difficulty in introducing new or modified functions (e.g. stimuli-sensitivity, biodegradation, and biorecognition) [21, 22] .

Recombinant DNA technology has allowed the biological synthesis of engineered protein polymers containing repeating blocks of amino acids with precise composition, sequence, and length [23]. Control over the detailed physicochemical properties of the polymers, using recombinant DNA techniques, has important implications in the design of novel biomaterials for controlled gene delivery and other biomedical applications [24]. Precise control over polymer structure using recombinant techniques allows the development of novel gene delivery systems where polymer structure can be correlated with function (i.e., gene transfer). Table 2 outlines the advantages that recombinant cloning and expression of protein-based polymers provide over naturally occurring and chemically synthesized polymeric biomaterials. Genetically engineered protein-based polymer include but are not limited to elastin-like polymers, silk-like polymers, silk-elastinlike protein polymers (SELPs), poly (glutamic acid ) polymers and alanylglycine polymers [23]. Protein-based polymers can be designed to incorporate a variety of functionalities, including responsiveness to microenvironmental stimuli, controlled biodegradation, and the presentation of informational motifs for cellular and subcellular interactions. Biologically synthesized polymers do not contain toxic monomer residues and solvents and, depending on their structure, can biodegrade to nontoxic amino acids

and be eliminated at controlled rates from the body. However, limitations in cloning and expression especially for cationic polymers useful in gene delivery must be overcome before the successful evaluation of these systems as systemic gene carriers [25].

### **Polymers for systemic gene delivery and controlled release**

Most non-viral polymeric gene delivery systems are prepared by condensation of plasmid DNA with cationic vectors to form a dense core of DNA coated with a hydrophilic surface. The transfection efficiency of polymeric gene delivery systems after systemic administration depends on several factors including the physicochemical characteristics of the DNA/polymer complexes such as size and net charge, and presence of targeting, endolytic, and nuclear localization moieties among other factors. In the past decade, many polymers have been investigated for controlled delivery of genes to target cells. These include, but are not limited to, cationic poly (amino acid)s [26], poly vinyl pyrrolidone [27] and chitosans [28]. Both in terms of *in vitro* transfection efficiency of DNA / polymer complexes and *in vivo* biodistribution and gene expression, significant findings have been achieved. Despite successes in the design and testing of nonviral gene delivery systems, much work needs to be done to correlate structure with function. This is particularly important for the design of novel polymeric gene carriers. For example recently we studied the effect of the feed comonomer composition of a series of closely related random amino acid-based copolymers on the physicochemical properties of their complexes with plasmid DNA and the corresponding protection against degradation by nucleases, cytotoxicity and transfection efficiency at various DNA: polymer ratios [26]. Copolymers of lysine and serine showed protection of DNA against nuclease degradation

to a higher extent compared to other copolymers of lysine and alanine or arginine and serine. The presence of arginine residues increased the transfection efficiency of the complexes by 2-3 orders of magnitude, comparable to that of the standard transfection agent Lipofectamine (Figure 1). Though these studies provide insight in the influence of amino acid composition on transfection, due to the random nature of these copolymers and heterogeneity in molecular weight it is difficult to establish concise polymer structure /transfection relationships. Ongoing work involves engineering better defined polymers using recombinant techniques to delineate the influence of sequence, molecular weight and composition on transfection efficiency.

An alternative route of administration is direct intratumoral injection using controlled release polymeric delivery systems. Matrix-mediated gene delivery systems provide several advantages over bolus administration such as the ability to manipulate the release profile to localize the delivery of the transgenes to the target tissue in a sustained and predictable manner, protect DNA from endogenous nucleases when encapsulated in a matrix and delivery of more than one agent at a time for combination therapy. Hydrogel-forming genetically engineered silk-elastinlike protein polymers (SELPs), containing four silk and eight elastin units in the repeat monomer with one elastin unit containing a lysine substitution, have been investigated in our laboratory as matrices for controlled gene delivery [25, 29, 30]. Results show that the rate of DNA release from these systems is affected by variation in the ionic strength of the media, hydrogel cure time and polymer concentration [30]. The release of plasmid DNA from the gels fluctuated with changes in the ionic strength of the medium in an "on / off" fashion, which was attributed to the presence of one cationic lysine residue in each monomer unit of the polymer backbone.

Plasmid release from recombinant SELP matrices is both DNA conformation- and size-dependent [29]. The cumulative release of the three DNA conformations was in the order of linear > supercoiled > open circular, which is assumed to be due to differences in topology of the three conformations and therefore their contact with the hydrogel network. Whereas, the release of a series of plasmids with different sizes showed that the smallest plasmid, pUC18 (2.6 kbp) was released fastest, followed by pRL-CMV (4.08 kbp), pCFB-EGSH-luc (8.5 kbp), and the largest plasmid pFB-ERV (11 kbp), which showed less than 5% release over 28 days (Figure 2). The size-dependent release of plasmid DNA is likely due to the hindered diffusion of larger plasmids through the pores that are formed by physical crosslinking of the hydrogels.

*In vitro* release studies showed that plasmid DNA remains structurally intact with regard to molecular weight and conformation over 28 days [29]. *In vivo* delivery evaluated by delivering pRL-CMV, containing the *Renilla* luciferase gene, intratumorally in a murine model of human breast cancer showed that tumor transfection was significantly enhanced for up to 21 days when compared to the delivery of naked pRL-CMV in a hydrogel composition-dependent manner [29].

In addition to delivery of naked DNA, recent efforts have explored the localized matrix-mediated delivery of viral vectors [21, 22] taking advantage of the greater transfection efficiency provided by the adenoviruses. Recombinant protein-based polymers provide a platform where viral vectors can remain viable for prolonged periods while polymer structure can be tailor-made to achieve desirable release and degradation profiles. The *in vitro* release and bioactivity of adenovirus containing the green fluorescent protein (gfp) gene, as a marker of gene transfer, for up to 22 days from



hydrogels have shown a clear qualitative relationship between the percentage of polymer in the hydrogel and transfection *in vitro*, indicating that the release of adenoviruses can be modulated by changing the composition of the matrix [29].

The simplicity of loading plasmid DNA or viral vectors into recombinant SELP hydrogels by mixing, the mild conditions under which the gel formation can be controlled (absence of organic solvents and extreme pH, etc.), and the ability of SELP hydrogels to deliver plasmid DNA and adenoviral vectors *in situ* after injection through a needle suggest that these materials have potential in applications requiring controlled release of DNA or viral vectors.

The use of recombinant polymers for controlled drug and gene delivery is in its infancy [23, 25]. The full potential of genetically engineered polymers in cancer gene therapy will be realized by further systematic polymer structure / delivery relationships where protection of the cargo (plasmid DNA, viral particles, etc.) from degradation, targeting to the desired site of action, release from endosomal compartments and localization at the nucleus can be achieved [20]. In addition issues surrounding the cloning and expression of cationic polymers, biocompatibility, biodegradation and elimination, and high initial cost, among other things, need to be addressed.

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Dr. Ghandehari obtained both his BS in Pharmacy (1989) and PhD in Pharmaceutics and Pharmaceutical Chemistry (1996) from the University of Utah. Research in his laboratory focuses on the development of novel methods of controlled delivery using polymeric biomaterials. Mohamed Haider obtained his B. Sc. Degree in Pharmacy (1997, Cairo University). In 1999, he joined the graduate program of the Department of Pharmaceutical Sciences at the University of Maryland at Baltimore. His dissertation focuses on controlled gene delivery using amino acid - based polymers.

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Table 1. Diseases addressed by gene therapy clinical trials\*

<b>Diseases</b>	<b>Protocols</b>	
	<b>Number</b>	<b>%</b>
Cancer	403	68.8
Monogenic diseases	78	13.3
Infectious diseases	41	7
Vascular diseases	51	8.7
Other diseases	12	2%
Total	585	100

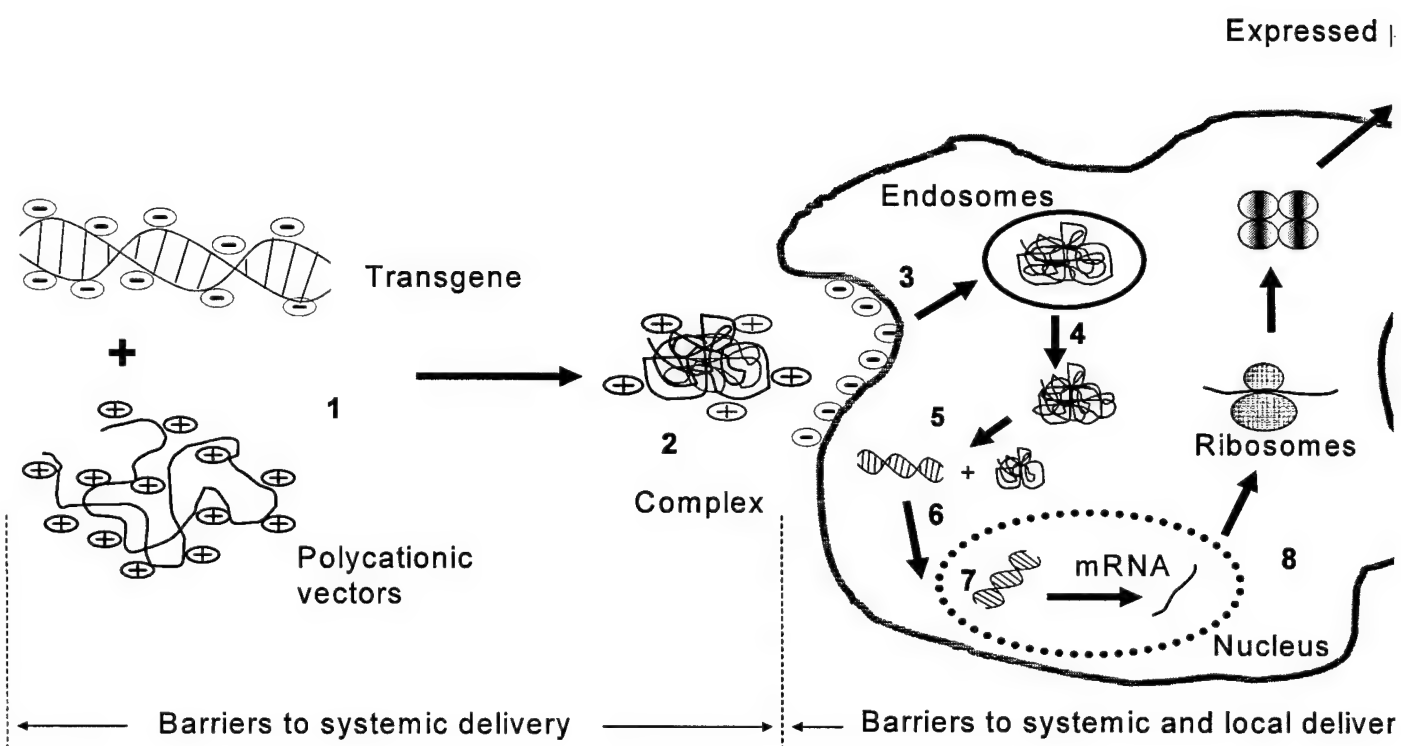
\*Adapted from reference [1] with permission.

Table 2. Comparison between chemical synthesis and genetic engineering of polymers

<b>Chemical Synthesis</b>	<b>Genetic Engineering</b>
<ul style="list-style-type: none"> <li>• Polydisperse</li> <li>• Monomer sequence generally not well-defined</li> <li>• Difficult to achieve stereoregularity</li> <li>• Difficult to incorporate motifs from nature</li> <li>• Initial production generally requires lower costs</li> <li>• Procedure can be standardized</li> </ul>	<ul style="list-style-type: none"> <li>• Theoretically monodisperse</li> <li>• Well-defined monomer sequence</li> <li>• Polymers obtained are stereoregular</li> <li>• Ability to incorporate motifs from nature</li> <li>• Expensive initial design and production</li> <li>• Depends on biological systems that are difficult to standardize</li> </ul>

### **Scheme legend.**

Scheme 1. Barriers to systemic and local gene delivery: (1) complex formation and characterization, (2) transport in blood circulation, (3) uptake/entry into the cell, (4) release from endosome, (5) dissociation from synthetic vector, (6) transit from cytoplasm to nucleus, (7) uptake/entry into nucleus and (8) transgene expression.



**Scheme 1**

## Figure legends

**Figure 1.** Transfection of Cos-7 cells after incubation with plasmid DNA / polymer complexes at different DNA: polymer wt/wt ratios: (◆) poly [(Lys, Ala) 1:1], (✱) poly [(Lys, Ala) 2:1], (▲) poly [(Lys, Ala)] 3:1, (✕) poly [(Lys, Ser) 3:1], (■) poly [(Arg, Ser) 3:1], (●) Poly-L-Lys. (Values are reported as average of  $n = 9 \pm \text{SD}$  where  $n$  represents the number of times each complex was made and transfection efficiency determined). From reference [27] with permission.

**Figure 2.** Effect of plasmid DNA size on *in vitro* release from recombinant SELP-47K hydrogels: (■) pUC 18 [2.6 Kbp] (✕) pRL-CMV [4.08 Kbp], (●) pCFB-EGSH-Luc [8.5 Kbp], (▲) pFB-ERV [11 Kbp]; (—) Theoretical release. Gel dimensions:  $r = 2.3 \text{ mm}$ ,  $h = 2.8 \text{ mm}$ ,  $v = 50 \mu\text{l}$ . Each data point represents the mean  $\pm$  standard deviation for  $n=3$  samples. From reference [30] with permission.

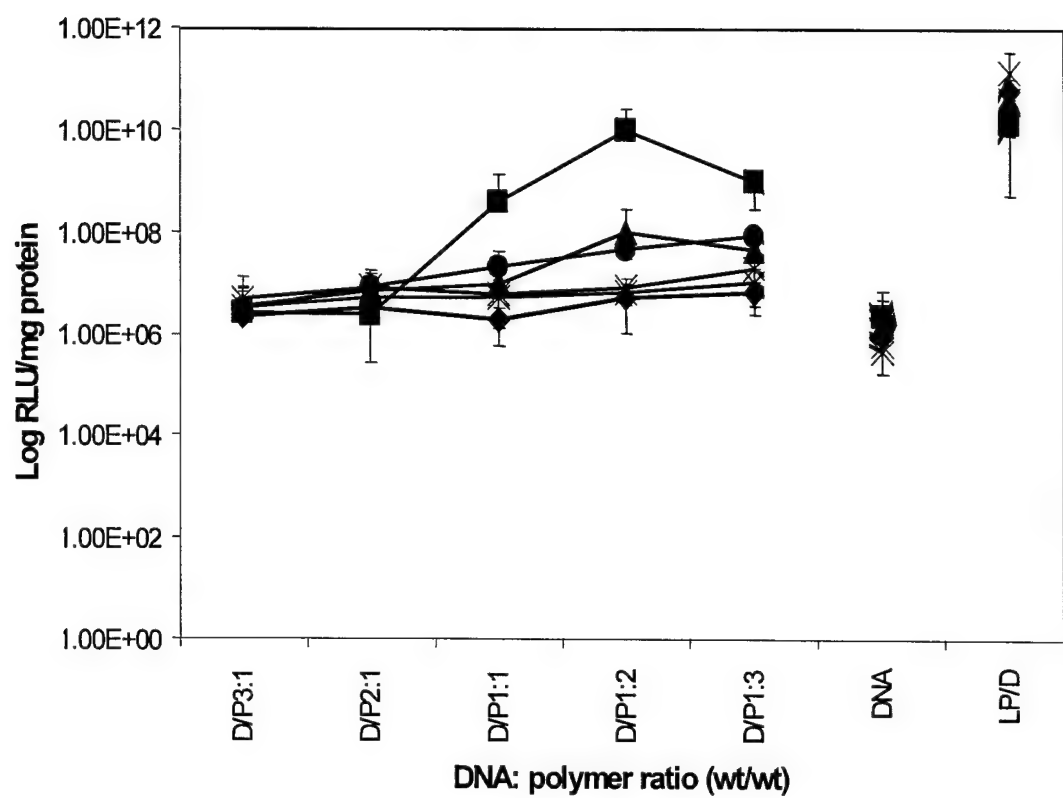


Figure 1

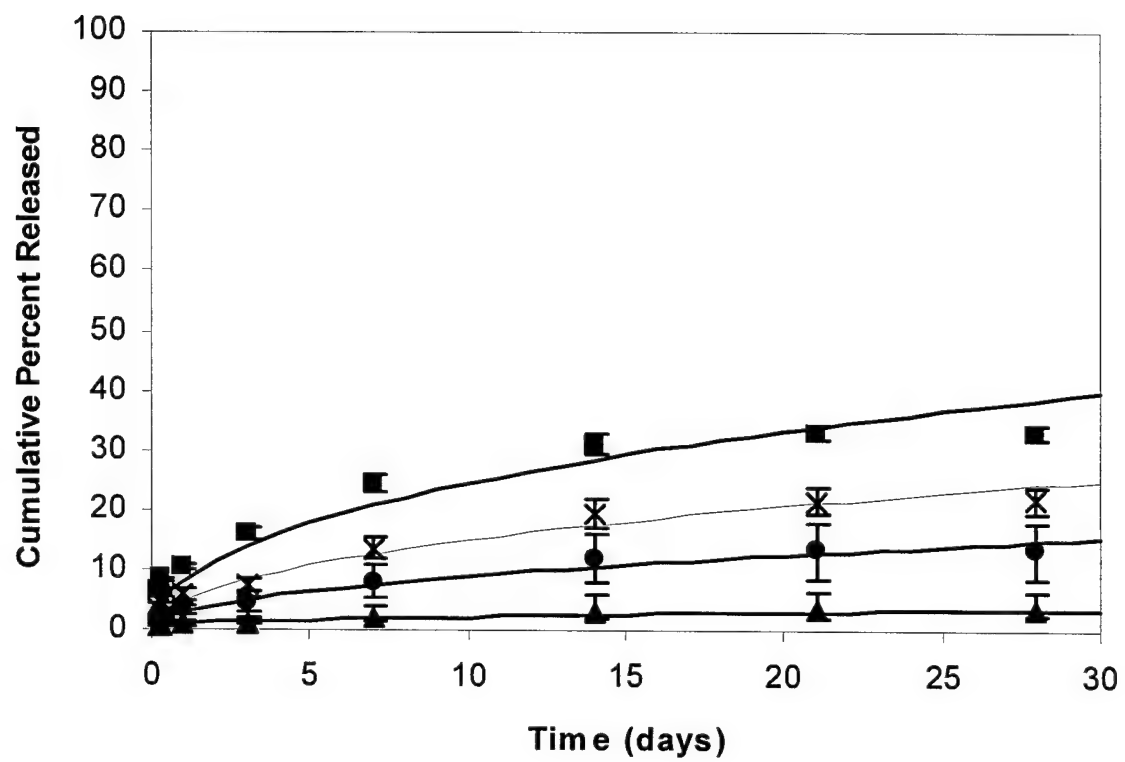


Figure 2



## **Genetically Engineered Protein-Based Polymers: Potential in Gene Delivery**

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## Introduction

The relatively recent explosion of information on protein structure and function has led to a shift in how proteins are perceived. Detailed structural information, obtained by techniques such as x-ray crystallography, have provided an intricate picture of the conformational properties that underlie protein function. Coupling of this structural information with molecular modeling and physical manipulation techniques, such as steered molecular dynamics simulations and atomic force spectroscopy, has enabled the detailed characterization of proteins' structural and biochemical responses to stimuli such as applied stress [1, 2]. In parallel with these molecular characterizations of protein structure and function, an interest has developed in the synthesis and characterization of protein-based materials, or *materials biotechnology*. While scientists have long studied the material properties of structural proteins, such as silk, elastin, collagen, and keratin, it is only more recently that the interesting material properties of other proteins, and their subunits, are being recognized. The extensive mechanical characterization of the giant muscle protein titin, and its immunoglobulin and fibronectin subunits, is one example [3].

As the material properties of protein subunits are further characterized, applications for protein-based materials are emerging in several fields, including the biomedical arena. The purpose of this chapter is to explain the rationale behind the utilization of protein-based biomaterials, their synthesis and characterization, and biomedical applications including gene delivery. Though applications specifically intended for gene delivery have just begun to emerge, the perceptive reader will be able to extrapolate the potential for gene delivery applications rather easily.

## Genetically Engineered Protein-Based Polymers

Genetically engineered protein-based polymers (hereafter referred to simply as *genetically engineered polymers* or *protein-based polymers*) are a class of polymers that can potentially include poly (amino acid)s, natural proteins and/or protein domains, and hybrids of these to produce artificial proteins. The key distinction that separates this class of materials from chemically synthesized polymers is that the composition, sequence, and molecular weight of genetically engineered polymers are programmed by recombinant techniques at the DNA-level [4]. By contrast, *sequential polypeptides* are synthesized by chemical oligomerization of short amino acid sequences, often synthesized by solid-phase techniques. *Chemically synthesized poly (amino acid)* homopolymers or copolymers are generally synthesized by random chemical reactions of a homogeneous or heterogeneous pool of amino acids. The synthesis of both sequential polypeptides and poly (amino acid)s results in a mixture of products with a distribution of molecular weights. Though sequential polypeptides offer a limited degree of control over amino acid sequence and composition, poly (amino acid) random copolymers have a random sequence, and their composition depends on the reactivity of the monomers. Recombinant methods allow the construction of DNA templates encoding protein-based polymers that can be utilized in a number of expression systems, including bacteria, fungi, and plants [5-8].

### *Synthesis and Characterization of Genetically Engineered Polymers*

Although there are several synthetic strategies for the production of genetically engineered polymers, all rely on the basic principle of self-ligation (concatamerization) of

DNA monomers to form concatameric DNA sequences [9, 10]. These polymeric genes are then transcribed and translated by the cellular machinery to produce a protein-based polymer. The synthesis of genetically engineered polymers begins with the conceptual design of an oligonucleotide sequence encoding the desired monomer. When designing this sequence, there are several biological constraints that must be balanced with the desire to make a given material: First, the codon usage preference of the organism in which the polymers are to be synthesized (usually *Escherichia coli*) must be considered. Repetitive usage of rare codons can lead to transfer RNA (tRNA) depletion, completely inhibiting the synthesis of some polymers and causing truncation of others. Second, while polymeric gene sequences require repetition by nature, the repetition of identical codons should be minimized. The redundancy of the genetic code can be exploited to achieve this objective while simultaneously maintaining a repetitive amino acid sequence. The consequences of highly repetitive codon usage include tRNA depletion and genetic instability. Some organisms, including *E. coli*, truncate repetitive genes through recombination or deletion [11-13]. Genetic instability arising from recombination or deletion may make it difficult or impossible to produce polymers from some DNA sequences. Third, the sequence should be designed to minimize complementarity that can cause secondary structure formation at the messenger RNA (mRNA) level. Formation of mRNA secondary structures can lead to pausing and disengagement of the ribosome during translation of the mRNA, leading to truncated polymers.

After designing the monomer oligonucleotide sequence, it is synthesized by automated chemical synthesizers. The oligonucleotide size limit allowed by the current

technology is approximately 100 bases. This limit sometimes requires the synthesis of multiple oligonucleotides, which can be enzymatically ligated to form monomers with a length greater than 100 nucleotides. After synthesis, the monomers are purified and annealed with their complementary strands to make double-stranded DNA that is suitable for cloning.

During synthesis, two types of plasmids (*vectors*) are frequently used. These are the *cloning* vector and the *expression* vector. The cloning vector is a plasmid that lacks the DNA sequences necessary for transcription of an inserted gene. Cloning vectors are normally used to “store” genes in a stable form until they can be placed in expression vectors. Expression vectors contain the DNA sequences necessary for gene transcription, and in combination with a complementary cell line can be used to express a protein (polymer) of interest. To minimize genetic instability early in the process, a cloning vector is often used in the first stage of polymer synthesis. The monomer gene is inserted into the vector and the vector is transformed into *E. coli*, which can be grown to produce large quantities of the vector and hence the monomer gene that it contains. The presence of the monomer gene in the vector is confirmed by restriction digestion and/or the polymerase chain reaction (PCR) and its sequence is confirmed by DNA sequencing [14].

After the presence and identity of the monomer gene have been confirmed, DNA concatamers are synthesized. The first step in the synthesis of concatamers is the production of relatively large amounts of monomer. This can be done either by performing large-scale plasmid preparations or by PCR. The classic technique for concatamerization of DNA monomers involves the incubation of monomers with ligation enzymes such as T4 DNA ligase. This strategy produces an assortment of DNA

concatamers in which the size can be roughly controlled by the reaction time and the concentration of the monomers. However, this technique suffers from three primary limitations: First, because of the random nature of the ligation reaction, there is no guarantee that a concatamer of a desired size will be obtained. Second, as concatamer size increases, circularization can occur, preventing ligation with a vector. Third, the method requires the inclusion of unique restriction enzyme recognition sites that may require the insertion of extraneous codons (and hence amino acids) between monomers. These three limitations have been addressed by the development of slightly altered synthetic strategies. Recursive directional ligation is a method that can be used to synthesize concatameric DNA sequences of precisely defined length while avoiding circularization problems [15]. Another technique, relying on the isolation and ligation of pre-ligated concatamers of defined length, has also been utilized to control length and limit circularization [16]. Finally, a technique, termed *seamless cloning*, takes advantage of the fact that type II restriction endonucleases remove their own recognition site, to produce monomers and concatamers without any extraneous codons between monomers [17, 18].

After synthesis, the DNA concatamers are ligated into either another cloning vector or an expression vector. The choice of the vector is partially based upon the availability of the necessary restriction sites and desired purification tag, and will vary depending on the cloning strategy. When the polymer gene is cloned into an expression vector, it is usually transformed first into a strain of bacteria that is incapable of expressing it. As with the use of a cloning vector, this approach avoids potential problems related to genetic stability and/or cytotoxicity due to basal levels of polymer

expression. However, the production of polymers eventually requires that an expression plasmid containing the polymer gene be transformed into an expression host.

Most *E. coli* expression systems in use today contain an inducible promoter that can be used to turn on polymer production once the cells have grown to an optimal density. This strategy is intended to maximize the efficiency of polymer production. Production of recombinant proteins redirects metabolic resources that are normally used for growth, maintenance, and division, often leading to a slowing or arrest of growth. By allowing the cells to reach a relatively high density prior to inducing polymer expression, the need for further division can be somewhat circumvented and the yield of the polymer can be increased.

Purification of polymers is performed by standard techniques that have previously been established for recombinant proteins. Frequently these include the use of affinity tags (e.g., poly (histidine), glutathione-s-transferase) that can be used to chromatographically purify recombinant proteins. If necessary, the tag can subsequently be removed by enzymatic cleavage. Some polymers have been purified on the basis of their physicochemical properties. For example, silk-like polymers have been purified by taking advantage of their low solubility in aqueous medium [19]. Elastin-like polymers (ELPs) have been purified by temperature cycling above and below their inverse temperature transition ( $T_i$ ) [20]. This technique has been extended to produce an ELP-tag that can be used to purify a number of recombinant proteins by temperature cycling, which is faster and less expensive than column chromatography [21].



After purification, polymers are typically characterized by a standard set of techniques that can include amino acid content analysis, mass spectrometry, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting. These methods are intended to verify the identity of the polymer. Depending on the type of polymer being synthesized, a series of polymer-specific characterizations will then be performed. For example, ELPs are typically characterized in terms of their  $T_i$ .

While biological synthesis of polymers confers many advantages, it also imposes some limitations. As described previously, there are several issues that must be considered when designing the monomer and concatamer gene sequences. Furthermore, the potential toxicity of the genetically engineered polymer to the expression host, and the resultant effect on the product, must be considered. As discussed later, highly charged proteins may be particularly problematic.

While the 20 natural amino acids do provide significant structural diversity, the natural amino acid pool can also be viewed as a limitation. Kiick *et al.* have begun to address this limitation by incorporating artificial amino acid analogs into genetically engineered polymers [22]. Incorporation of the amino acid azidohomoalanine into the medium of methionine-depleted bacterial cultures resulted in the replacement of natural methionine with azidohomoalanine. The significance of this substitution is that it allows chemical modification of the azide group by the Staudinger ligation.

## Biomedical Applications of Genetically Engineered Polymers

### *Biocompatibility and Biodegradation*

The fact that genetically engineered polymers are composed of naturally occurring amino acids, and often naturally occurring protein motifs, raises the possibilities that these polymers could be designed to be enzymatically biodegradable and responsive to physiological stimuli. The insertion of proteolytic enzyme recognition sequences into the polymer backbone allows for fine control over the rate of biodegradation of protein-based polymers, which are thought to eventually degrade to their amino acid constituents [23, 24]. However, these desirable attributes must be tempered by the possible immunogenicity of some protein sequences and/or their degradation products.

The silk-elastinlike polymers (SELPs) are one class of genetically engineered polymers for which biocompatibility and biodegradation have been extensively characterized. SELPs are a family of copolymers with the general structure  $[(S)_x(E)_y]_z$ , where (S) is a motif from *Bombyx mori* (silkworm) silk (Gly-Ala-Gly-Ala-Gly-Ser) and (E) is a motif from mammalian elastin (Gly-Val-Gly-Val-Pro) [5]. Studies of implanted SELP films have shown a mild, localized immune response with some macrophage surveillance up to approximately one week [23]. After one week, the implanted SELP films were observed to be surrounded by fibroblasts and collagen, indicating that healing was occurring, while the number of macrophages around the implant decreased. Implanted SELP sponges were infiltrated by fibroblasts, indicating a high level of biocompatibility with living cells. Similar results were observed in a model of wound healing. Rabbits injected with SELPs were found to develop antibodies to the silk-like

blocks of the polymer, but not to the elastin-like blocks. Biodegradation of SELPs was found to occur in a manner influenced by their composition *and* sequence [23].

### *Applications of Genetically Engineered Polymers*

Genetically engineered polymers have been used for early-stage studies in several applications of biomedical interest. Perhaps the class of polymer most widely studied has been the ELPs. Chemically synthesized sequential ELP polypeptides and their genetically engineered counterparts have the general sequence (Gly-Xaa-Gly-Val-Pro), where (Xaa) is a substitutable residue that can be used to tailor the properties of the ELP. ELPs exhibit a phase transition that can be triggered by changes in ionic strength, pH, and polymer concentration. Hence, by varying the molecular weight and (Xaa), ELPs can be designed to precipitate under a variety of environmental conditions [25].

Cappello *et al.* have synthesized silk-like polymers that contain periodically spaced fibronectin sequences [26, 27]. These polymers, called SLP-F, have been shown to support the attachment of a variety of cell types, including epithelial cells, fibroblasts, and cancer cells [26]. In addition, silk-like polymers containing regions of human laminan have been synthesized (SLP-L) [23]. The SLP-L polymers promoted greater attachment and spreading of fibrosarcoma and rhabdomyosarcoma cell lines than polylysine or laminan coatings. The inclusion of these attachment sequences in-between silk-like blocks allows them to withstand autoclaving without loss of activity [23]. Similarly, Panitch *et al.* have incorporated periodically spaced fibronectin CS5 domains into ELPs [28]. These polymers enhanced the attachment of endothelial cells to a glass

substrate. Genetically engineered polymers containing cellular attachment motifs could be promising materials for tissue engineering and applications requiring the delivery of genes from surfaces.

Halstenberg *et al.* have synthesized hybrid protein-*graft*-poly (ethylene glycol) polymers for tissue repair [24]. The engineering of this biomaterial was particularly elegant, as it included motifs for rationally controlling both cellular attachment and biodegradation. Hydrogels prepared by crosslinking the polymer acted as scaffolds for three-dimensional outgrowth of fibroblasts, which attached to the polymer via specific interactions between cellular integrins and an RGD sequence on the polymer. Outgrowth involved serine protease degradation of the polymer at sites engineered for this purpose.

Localized hyperthermia has been used to target systemically administered ELPs [29]. ELP sequences were chosen so that the  $T_i$  of the polymer (40 °C) was above body temperature (37 °C) but below a temperature induced by localized heating of tissue (42 °C). Using this technique, rhodamine-ELP conjugates were observed to accumulate in ovarian tumors by *in vivo* fluorescence microscopy.

ELPs have also been investigated as biomaterials for cartilaginous tissue repair [30]. Chondrocytes cultured in ELPs retained their phenotype and synthesized significant amounts of extracellular matrix molecules that are important for cartilage function. This highlights the potential use of genetically engineered polymers as biocompatible extracellular matrices for tissue repair and engineering. It may also be possible to incorporate drugs and/or genes into these systems to guide the development of new tissue.

Recently, ELPs have been used for the patterning of proteins on surfaces. ELP fusion proteins self-assemble onto hydrophobic surfaces above  $T_i$  and desorb below  $T_i$  [31]. In combination with hydrophilic surfaces, which do not adsorb ELP, this technique can be used to create micropatterned surfaces for cell growth and protein arrays.

Hydrogels are crosslinked, water-swollen, polymer networks that have numerous biomedical applications, including the controlled delivery of genes [32, 33]. Several genetically engineered polymers have been synthesized that can form hydrogels through either physical or chemical crosslinking. Those forming hydrogels by physical crosslinking include polymers based on the leucine zipper motif [34] and some SELPs [35, 36]. Polymers containing the leucine zipper motif reversibly self-assemble in response to changes in temperature and pH [34]. Some SELPs (e.g., SELP-47K, Figure 1) irreversibly self-assemble via hydrogen bonding between their silk-like blocks [35, 36]. The release of solutes such as drugs, proteins, DNA, and macromolecular probes from SELP hydrogels have been investigated [33, 35, 37]. ELP-based hydrogels have also been fabricated. These include chemically crosslinked gels [38] and thermally reversible micellar aggregates that act as virtual crosslinks formed between elastin domains [39]. ELP-based hydrogels have been observed to phase or volume transitions in response to changes in temperature [40, 41].

Thermoreversible nanoparticles have been produced from ELPs with hydrophilic and hydrophobic blocks [42]. These nanoparticles were found to form a micellar core-shell structure, with the formation of the nanoparticles regulated by the dehydration of the hydrophobic block above the  $T_i$ . Dynamic light scattering studies indicated that the size of the nanoparticles was dependent on the temperature of the medium. There is

significant interest in the synthesis of polymeric micelles for gene delivery [43]. Genetic synthesis of micelle-forming polymers that are well-defined and responsive to stimuli may enhance their effectiveness for some gene delivery applications.

Another class of related biomaterials combines genetically engineered polymers with chemically synthesized polymers (i.e., *hybrid* polymers). Combining these two synthetic techniques allows the synthesis of a broad range of materials that is not accessible by either means alone. Some examples of materials synthesized by these techniques include *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers crosslinked by coiled-coils and immunoglobulin domains [44, 45]. Both materials form hydrogels that are responsive to temperature, with the former collapsing with increased temperature, while the latter expands. These hydrogels could potentially be useful for thermally controlled gene delivery applications. The previously discussed protein-graft-poly (ethylene glycol) polymers are another example of a hybrid polymer [24].

### **Gene Delivery Applications for Genetically Engineered Polymers**

In order for gene therapy to be successful as a treatment, gene delivery must first reliably overcome a series of biological barriers. These barriers are discussed in detail in other chapters of this book. The research in our laboratory is currently focused on overcoming biological barriers on two levels: (1) site-specific controlled delivery of naked DNA and viral vectors, and (2) condensation of DNA and endosomal escape at the intracellular level. Our results from the first branch of research will be discussed and the rationale and strategies for approaching the second problem will be presented.

One significant problem with current gene delivery systems is targeting them to the desired site of action. Targeting can improve therapeutic benefit and reduce toxicity, by increasing the concentration of the vector at the site of action while simultaneously minimizing the delivery of the vector to other parts of the body. The targeting strategy is dependent on the route of administration: *systemic* or *site-specific*.

Targeting after systemic administration can be broadly categorized as *active* or *passive*. Active systemic targeting refers to targeting of a gene delivery system via a specific molecular interaction (e.g. between a motif on the vector and a receptor in the targeted tissue). Ligands used for active targeting include antibodies [46], transferrin [47], folate [48], and peptides [49], among others. Ideally, actively targeted vectors accumulate only in the targeted tissue. In reality, active targeting is complicated by the fact that cellular receptors and ligands are often broadly expressed on many different cell types. This can lead to significant delivery of the vector outside of the intended tissue.

Passive systemic targeting relies on the intrinsic, non-specific relationship between the physicochemical properties of a molecule (or vector) and the systemic physiology. Targeting ligands and receptors are not used in passive targeting. The enhanced permeability and retention effect, causing accumulation of macromolecules in solid tumors, is one example of passive targeting [50].

In contrast to systemic targeting, site-specific controlled delivery targets a specific location by implantation of and release from a matrix or device. An example of this approach is controlled gene delivery from polymeric matrices [51-55]. The use of polymeric matrices to deliver genes confers three primary advantages over bolus



administration: First, the release profile of the gene can be manipulated by altering the physicochemical properties of the polymer and fabrication of the matrix. This allows gene delivery to occur in a prolonged, sustained, and controlled manner, possibly increasing its effectiveness. Second, encapsulation of DNA or a vector in a polymeric matrix may offer some protection against degradative enzymes such as nucleases and proteases. Third, polymeric matrices allow precise spatial localization, obviating the need for active targeting ligands that may be required for systemic administration. Our research has focused on investigating the potential of genetically engineered SELPs as *in situ* gel-forming matrices for the controlled *intratumoral* delivery of plasmid DNA and adenoviral vectors [33, 56].

Given the current limitations of actively targeted, systemic cancer gene delivery systems, intratumoral delivery offers a logical alternative. The primary advantages of intratumoral gene delivery stem from its very precise spatial localization. Direct injection into the tumor obviates the need for active or passive targeting on the systemic level. The efficiency of the therapy may be further increased by attaching tumor-specific ligands to a vector [47]. The primary disadvantage of intratumoral gene therapy is that each tumor must be individually identified and injected. Hence, by virtue of the difficulty in their detection and distributed nature, small metastases could be impossible to treat by this method. Intratumoral cancer gene therapy may thus be best suited for the treatment of cancers that grow slowly (e.g., prostate cancer) and/or where potential complications or disability make tumor resection less desirable (e.g., prostate cancer, head and neck cancer).

### *Controlled Gene Delivery from Silk-Elastinlike Hydrogels*

One member of the SELP family, SELP-47K (Figure 1) forms hydrogels spontaneously through hydrogen bonding between the silk-like blocks. Formation of this physically crosslinked hydrogel does not require the use of organic solvents or toxic crosslinking reagents that may damage DNA or viruses, or cause cytotoxicity. While hydrogel formation is a kinetic process at any temperature, it is substantially accelerated at 37 °C versus room temperature, allowing polymer/DNA or polymer/virus solutions to be prepared at room temperature and to form matrices *in situ* within a few minutes of injection.

As a step toward gene delivery applications, the degree of swelling of SELP-47K hydrogels was characterized as a function of environmental conditions. The degree of swelling describes the amount of water imbibed by a hydrogel network. This parameter provides insight into the relative crosslinking density of the network and hence its porosity and solute transport characteristics. Swelling of SELP-47K hydrogels was insensitive to changes in ionic strength, temperature, and pH [36]. The insensitivity of SELP-47K hydrogels to changes in environmental conditions was explained by the immobilization of the elastin-like blocks through the irreversible self-assembly of the silk-like blocks. However, the degree of swelling of the hydrogel decreased with increasing polymer concentration and cure time [36]. These results are consistent with the increased network density expected from increasing the amount of polymer in the matrix and/or increasing the time allowed for matrix formation, since it is a kinetic process. SELP copolymers containing one silk-like block have been synthesized that exhibit responsiveness to pH, ionic strength, temperature, and concentration [57, 58].

These polymers do not form physically crosslinked hydrogels under conditions studied thus far but can form aggregated particles depending on the above mentioned conditions.

In order to assess its potential as a matrix for controlled gene delivery, the release of plasmid DNA from SELP-47K hydrogels was evaluated over 28 days [33, 56]. DNA-containing hydrogels were fabricated by mixing aqueous DNA solution with aqueous SELP-47K solution. DNA/polymer solutions were incubated at 37 °C to induce gelation.

DNA release was initially evaluated as a function of polymer concentration, DNA concentration, ionic strength, and cure time [33]. Increasing the polymer concentration and/or cure time decreased the rate of DNA release from the hydrogels, an effect consistent with an increased network density. Over the range studied, the DNA concentration did not influence the rate of release, with the fraction released at each time point being identical for hydrogels containing 50 or 250  $\mu\text{g/ml}$  DNA. The ionic strength of the medium strongly influenced the rate of DNA release, with virtually no release observed at ionic strengths below  $\mu=0.17$  M, while identical release profiles were obtained above this ionic strength, up to  $\mu=0.50$  M (Figure 2A). This ionic strength dependence was thought to arise from an ionic interaction between the positively charged lysine and arginine residues on SELP-47K (Figure 1, boxed) and the negatively charged DNA phosphates.

To verify that DNA release was modulated by the effect of buffer ionic strength on the polymer-DNA interaction, a turbidity study was performed that evaluated the interaction between DNA and SELP-47K in buffers with various ionic strengths. Polymer and DNA were mixed at various molar charge ratios and the turbidity of each solution was determined using a spectrophotometer, at 400 nm. This method permitted

the detection of insoluble interpolyelectrolyte complexes formed by electrostatic interaction between SELP-47K and DNA. As shown in Figure 2B, the relative turbidity of the mixtures showed a substantial increase when complexes were prepared in PBS with low ionic strength ( $\mu=0.03$ ), indicating that at this ionic strength insoluble complexes form between SELP-47K and DNA. No increase in turbidity was observed in the buffer with an ionic strength of 0.17 M, indicating that DNA and polymer do not interact at this ionic strength. These results are consistent with the observed release data (Figure 2A).

DNA release from the hydrogels was observed for at least 28 days, with apparent diffusion coefficients on the order of  $10^{-9}$  to  $10^{-10}$   $\text{cm}^2/\text{s}$ , depending on the polymer concentration and cure time [33]. Transfection studies were performed on COS-7 cells to evaluate the bioactivity of plasmid DNA containing the *Renilla* luciferase gene, after encapsulation in SELP-47K hydrogels for up to 28 days [56]. Figure 3 shows the results of these transfection assays, which indicate that polymer-encapsulated DNA retained *in vitro* bioactivity equivalent to stock DNA for at least 28 days.

The conformation of plasmid DNA, namely supercoiled, open circular, or linear has been hypothesized to play a role in its transfection efficiency. While conventional thinking has generally indicated a preference for supercoiled DNA, at least one study has shown that the delivery of linear DNA results in prolonged transgene expression *in vivo* [59]. Other work has shown that the conformation of DNA is largely irrelevant to the transfection efficiency *in vitro* and *in vivo* [60]. It is possible that the conformational requirements of plasmid DNA may depend on the delivery system, whether the delivery

is taking place *in vitro* or *in vivo*, and the concentration of nucleases at the site of delivery.

In order to evaluate the influence of conformation on DNA release from SELP-47K hydrogels, plasmid DNA predominantly in the supercoiled, open circular, and linear conformations were produced and their release from SELP-47K hydrogels was evaluated [56]. The linear form of plasmid DNA was released most rapidly from the hydrogels, followed by the supercoiled form (Figure 4). The open circular form was practically not released, probably due to its impalement on the polymer chains. The influence of plasmid size on release was also investigated (Figure 5), with size-dependent release observed for plasmids from 2.6 to 11 kilobases (kb) in size, from 10 wt% SELP hydrogels [56]. Since hydrogels form from SELPs with concentrations as low as 4 wt%, it is possible that plasmids larger than 11 kb could be delivered from SELP hydrogels. The ability to deliver larger plasmids would be advantageous for large genes. One limitation of viral vectors is the size of the transgene.

The effect of hydrogel geometry on DNA release was also studied by fabricating hydrogels in the form of cylinders and flat discs [56]. Disc-like hydrogels released DNA faster than their cylindrical counterparts (Figure 6). This was attributed to their larger surface to volume ratio and was accurately described by fitting the release data to an equation that describes two-dimensional diffusion from a cylinder with geometric considerations [61, 62].

While the applications of SELP-mediated controlled gene delivery are numerous, we have focused our efforts on controlled delivery to solid tumors. Our initial studies in this arena involved the intratumoral delivery of a reporter plasmid (*Renilla* luciferase) to

solid tumors in a murine (athymic *nu/nu*) model of human breast cancer (MDA-MB-435 cell line) [56]. The injected SELP-47K solutions contained either 4, 8, or 12 wt% polymer and 70  $\mu$ g DNA per 100  $\mu$ l injection. At predetermined time points, animals were euthanized, tumors homogenized, and luciferase expression was assayed. The Mann-Whitney test was used to compare between treatment groups.

Delivery of the *Renilla* luciferase plasmid from SELP-47K matrices resulted in significantly enhanced tumor transfection for up to 21 days when compared to naked DNA (Figure 7). In particular, delivery of the plasmid from matrices containing 4 or 8 wt% polymer resulted in enhanced transfection up to 21 days, while 12 wt% matrices enhanced transfection up to 3 days (Figure 7). These results are consistent with sustained delivery from the 4 and 8 wt% matrices and entrapment of the DNA within the 12 wt% matrix.

The levels of tumor transfection mediated by the three concentrations of polymer were statistically equivalent until 7 days, when the 4 and 8 wt% matrices were both more effective than 12 wt% (Figure 7). The greater transfection persisted until 21 days for 4 wt% polymer and 14 days for 8 wt% polymer. Overall, the delivery of DNA from 4, 8, and 12 wt% hydrogels resulted in a mean 142.4-fold, 28.7-fold, and 3.5-fold increase in tumor transfection, respectively, compared with naked DNA over the entire 28 day period.

In addition to the delivery of DNA that occurs within the tumor, it can be expected that some DNA will diffuse from the matrix into to the surrounding tissue. In order to evaluate this, transfection of the skin approximately 1 cm around the tumor was measured. The enhancement of delivery to the tumor was compared to the levels of

transfection in the tumors (Figure 7) and skin (Figure 8) at each time point and polymer concentration. While statistically significant differences were not detected between all compositions, the mean tumor transfection was 42.0, 27.2, and 4.6 times greater than skin transfection for 4, 8, and 12 wt% hydrogels, respectively, over the entire 28 day period. This is in contrast to a 1.3 fold difference between tumor and skin transfection for naked DNA.

#### *Controlled Release of Adenovirus from Silk-Elastinlike Hydrogels*

Despite the promising results obtained from delivering plasmid DNA from SELP hydrogels, the delivery of naked DNA results in relatively low transfection efficiency and thus has limited applications. For this reason, we sought to explore the potential of SELP-47K to act as a matrix for the controlled delivery of viral vectors. The use of viruses that integrate into the host genome (e.g., retrovirus) can ensure long-term expression of a transgene. However, as was recently observed in a study of  $\alpha$ -linked severe combined immunodeficiency, insertion of the viral DNA at or near an oncogenic regulation site can lead to the development of cancer [63]. Thus, there is also an intense interest in non-integrating viral vectors, such as adenovirus, which may have a higher margin of safety than integrating viruses. However, without integration into the host genome the duration of transgene expression is limited. A controlled release approach may extend the duration of transgene expression by continuously delivering adenovirus at the site of action. In addition, it is conceivable that the immune response to the virus may be modulated by encapsulation in a polymeric matrix and only allowing the release of very small quantities of virus in a given time. Current state of the art polymers used in



drug delivery do not provide the capability to deliver viable adenoviral vectors effectively over a prolonged periods of time. Coacervate microspheres of gelatin and alginate have shown poor encapsulation efficiency, low virus bioactivity, and poor virus release kinetics [64]. In the case of natural polymers such as collagen, control over the rate of release is complicated by the limited control over polymeric structure and crosslinking density [65, 66]. Genetic engineering techniques may allow the design and synthesis of new polymers with precisely defined architecture, where biodegradation and biorecognition can be controlled to release viable viral vectors at specific sites and rates, in response to local stimuli.

As a first step toward that goal, we evaluated the potential of genetically engineered SELP-47K hydrogels to act as matrices for the controlled delivery of an adenovirus containing the green fluorescent protein (gfp) gene (AdGFP) [56]. SELP-47K/AdGFP solutions were prepared at 4, 8, and 11.3 wt% polymer. The mixtures were allowed to gel and hydrogel discs were placed in a PBS release medium. At predetermined time points, release medium was collected and used to transfect HEK-293 cells. These cells contain a relatively high density of adenovirus receptors and are thus a good screening tool for the presence and bioactivity of adenovirus in the release medium. Transduction was observed up to 22 days with the viruses released from the 4 wt% hydrogel (Column 2, Figure 9). The number of transduced cells obtained with the viruses released from the 8 wt% hydrogel (Column 3, Figure 9) was less than that obtained from the 4 wt% hydrogel. The 11.3 wt% hydrogel did not release any detectable adenovirus after the first day (Column 4, Figure 9). This demonstrates that adenoviral release can be controlled over a continuum by controlling polymer composition from no release (11.3

wt% gel) to greater release (4 wt% gel). Control samples (viral particles without hydrogels in release media, Column 1 Figure 9) were bioactive until day 29 (with few gfp+ cells on day 29). However, as anticipated bioactivity decreased over time. We are now focused on quantifying the amount of adenovirus released, the proportion that is bioactive, and the *in vivo* biodistribution, efficacy, and toxicity of adenoviral particles delivered from SELP-47K. The long-term goal is to engineer polymers tailor-made for specific needs.

A derivatized genetically engineered polymer, Pronectin F, has recently been studied as a nonviral vector for gene delivery [67]. Pronectin F is a silk-like polymer containing one fibronectin segment between every nine silk-like repeats. This polymer was originally synthesized by Cappello *et al.*, and has been explored as a substrate to enhance cellular attachment to hydrophobic materials [27].

Hosseinkhani and Tabata cationized Pronectin F by reaction of ethylenediamine, spermidine, and spermine with the hydroxyl groups of the serine in the silk-like blocks. For comparison, similarly cationized derivatives of gelatin were prepared. When complexed with plasmid DNA at a weight ratio of 50 protein : DNA, all forms of cationized Pronectin F (Pronectin F+) induced the formation of particles with a slightly positive (~10 mV) zeta potential and a particle size of approximately 200 nm. Both the zeta potential and the particle size were a function of the amount of Pronectin F+ added to the DNA.

All three Pronectin F+ derivatives increased the transfection of rat gastric mucosal cells by a reporter (luciferase) plasmid in comparison to naked plasmid. The spermine derivative was found to be significantly more effective than the ethylenediamine and

spermidine derivatives. This was attributed to the higher buffering capacity of the spermine derivative, which was comparable to polyethylenimine. Furthermore, cellular attachment mediated by the Pronectin F+ derivatives, containing 13 RGD motifs, was found to be significantly greater than the cationized gelatin, which contained only one RGD motif. The uptake of plasmid DNA into the cells, as mediated by the three Pronectin F+ derivatives, was quantified using fluorescent labels. In all cases, the amount of plasmid uptake mediated by the Pronectin F+ derivatives was found to be greater than that mediated by cationized gelatin. This study shows the potential of biorecognizable genetically engineered polymers as soluble nonviral vectors and surface coatings for gene delivery.

#### **Future Potential of Genetically Engineered Polymers in Gene Delivery**

In addition to the localized delivery of naked DNA and adenoviral particles, genetic engineering techniques may have potential to produce well-defined polymers for systemic gene delivery. Chemically synthesized cationic polymers, with a distribution of compositions and molecular weights have been widely investigated in nonviral gene delivery. However, the influence of polymer structure on the physicochemical properties and hence transfection efficiency of the polymer/DNA complexes is poorly understood. Some studies have sought to systematically define these relationships, but are limited by the inherent limitations of chemical polymer synthesis and derivatization, such as random sequences, polydispersity, and fractured structures [68, 69].

The biological synthesis of cationic polymers is attractive in that it could provide a series of macromolecules with well-defined sequence, composition, and molecular

weights for DNA complexation and transfection experiments. However, recombinant synthesis of cationic polymers presents a special problem, as many cationic proteins are inherently toxic to bacteria [70]. In fact, some proteins secreted by cells in the immune system (e.g. eosinophil cationic protein) serve this very purpose [71]. To decrease the toxicity of genetically engineered cationic polymers to the expression system, several approaches may be evaluated, including the inclusion of fusion tags that decrease the toxicity of the polymer, and/or the use of tightly regulated expression systems such as the pLysS-containing expression hosts [72]. Another approach, which may circumvent some of the problems associated with the expression of large molecular weight cationic polymers, would be to attach a short cationic tail to a targeting moiety. Multiple cationic tails could then theoretically interact with, and condense DNA, while the targeting moiety would enhance its delivery to cells. The inclusion of endosomolytic peptides at various points in the linear polymer may also enhance the efficiency of the vector by promoting endosomal escape. This research is at its embryonic stages. The long-term objective is to synthesize polymers for which structure-transfection relationships can be defined *in vitro* and *in vivo*. Some initial attempts, in our laboratory, to clone multimer genes encoding such polymers have been described elsewhere [73].

### **Conclusion / Future Direction**

The potential of genetically engineered polymers in gene delivery has been demonstrated by the delivery of plasmid DNA and adenoviral vectors from SELP-47K [33, 56]. The ability to form hydrogel depots *in situ* after injection through a needle, and the lack of exposure to organic solvents are key advantages that SELP hydrogels hold

over several other matrix-mediated controlled gene delivery systems. Genetic engineering techniques enable precise control over polymer structure and therefore phase transitions, biodegradation, and biorecognition.

Genetically engineered polymers also hold promise for the synthesis of nonviral vectors for systemic / targeted administration. It may be possible to use recombinant methods to synthesize modular gene delivery components that contain well-defined sequences for condensation, targeting, endosomal escape, and nuclear localization. The synthesis of polymeric gene carriers by this technique would also offer a way to examine structure-activity relationships of cationic polymers with unprecedented fidelity.

As our knowledge about natural proteins continues to grow, new genetically engineered polymers will be synthesized. Some of these molecules will have no precedent in the chemically synthesized polymer arena and may be designed to interact favorably with the complex physiological environment encountered by a gene delivery system on its way from administration to transcription.

### **Acknowledgements**

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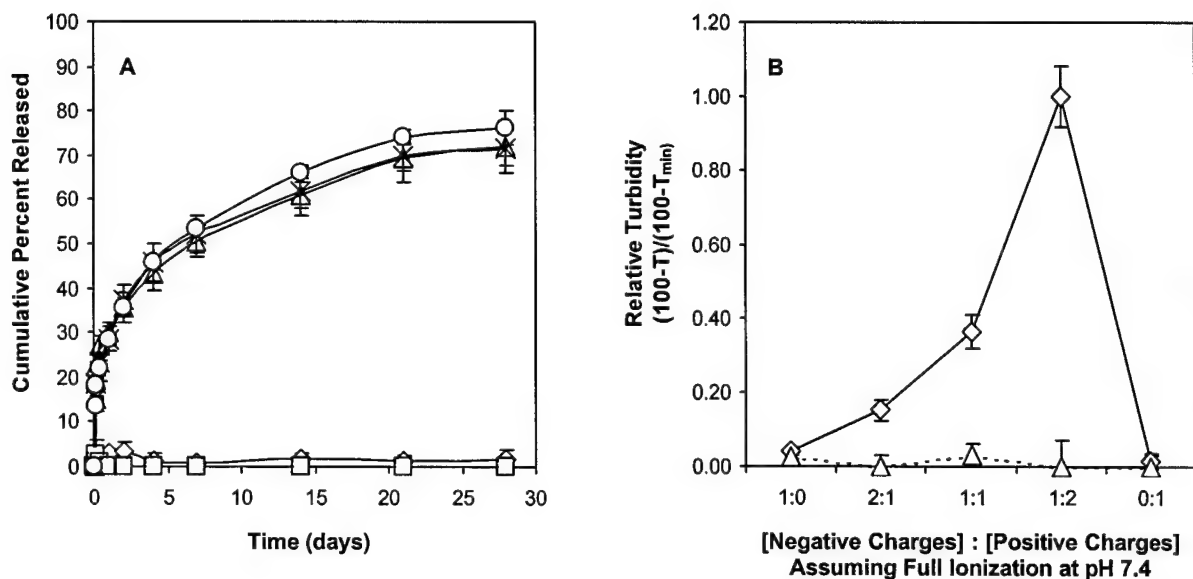
## Abbreviations

AdGFP	Adenovirus containing the green fluorescent protein gene
Ala (A)	Alanine
Arg (R)	Arginine
Asn (N)	Asparagine
Asp (D)	Aspartic acid
DNA	Deoxyribonucleic acid
ELP	Elastin-like polymer
gfp	Green fluorescent protein
Gln (Q)	Glutamine
Glu (E)	Glutamic acid
Gly (G)	Glycine
His (H)	Histidine
HPMA	<i>N</i> -(2-hydroxypropyl)methacrylamide
Leu (L)	Leucine
Lys (K)	Lysine
Met (M)	Methionine
mRNA	Messenger RNA
PBS	Phosphate buffered saline
Phe (F)	Phenylalanine
PCR	Polymerase chain reaction
pRL-CMV	<i>Renilla</i> luciferase plasmid
Pro (P)	Proline

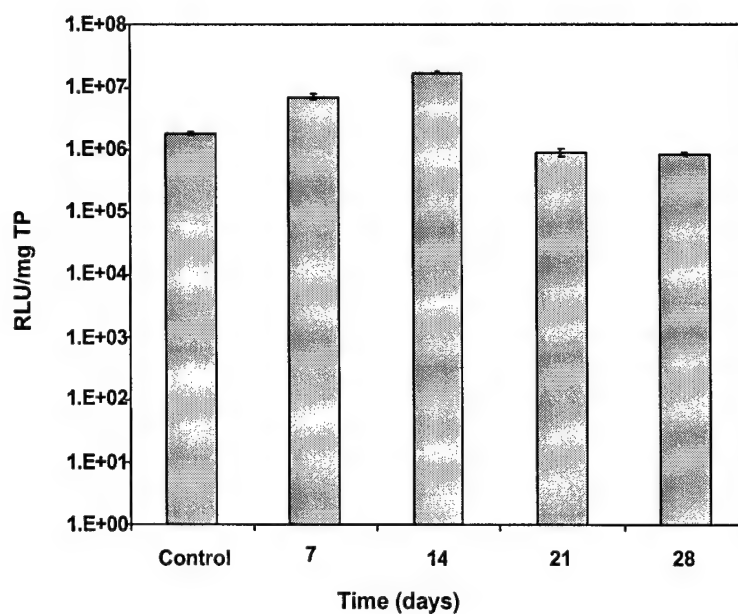
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SELP	Silk-elastinlike polymer
Ser (S)	Serine
Thr (T)	Threonine
tRNA	Transfer ribonucleic acid
Trp (W)	Tryptophan
T <sub>i</sub>	Inverse transition temperature
Tyr (Y)	Tyrosine
Val (V)	Valine
Xaa	A substitutable amino acid residue

MDPVVLQRRDWENPGVTQLNRLAAHPPFASDPM  
**GAGSGAGAGS**[(GVGV**P**)<sub>4</sub>GV**P**(GVGV**P**)<sub>3</sub>(GAGAGS)<sub>4</sub>]<sub>12</sub>  
 (GVGV**P**)<sub>4</sub>GV**P**(GVGV**P**)<sub>3</sub>(GAGAGS)<sub>2</sub>GAGA  
 MDPGRYQDLRSHHHHHH

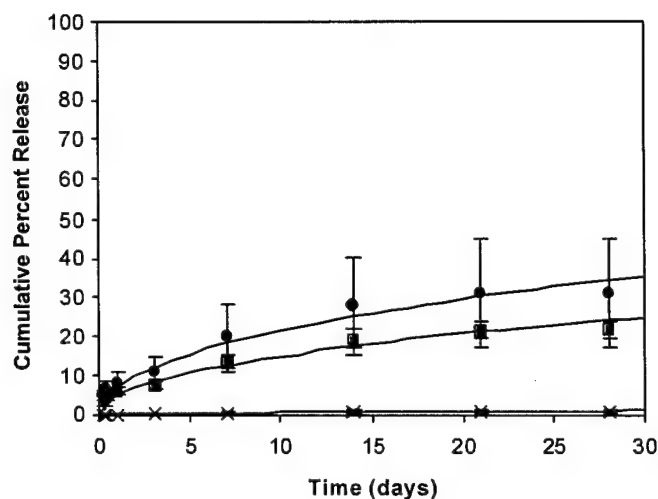
**Figure 1.** The 884 amino acid SELP-47K sequence has a molecular weight of 69,814 Daltons. It is composed of a head and tail sequence, and a series of silk-like (GAGAGS) and elastin-like (GVGV**P**) repeats. Residues that are predominantly positively charged at pH 7.4 are boxed.



**Figure 2.** (A) Cumulative release of pRL-CMV from 12 wt% SELP-47K hydrogels, in PBS with  $\mu=0.03$  M ( $\diamond$ ),  $0.10$  M ( $\square$ ),  $0.17$  M ( $\triangle$ ),  $0.25$  M (X), and  $0.50$  M ( $\circ$ ). Hydrogels were cured for one hour at  $37^\circ\text{C}$  before placement in the appropriate buffer. Each point represents average  $\pm$  standard deviation ( $n=3$ ). (B) Effect of ionic strength on the formation of insoluble complexes between SELP-47K and pRL-CMV, in PBS with  $\mu=0.03$  M ( $\diamond$ , solid line) and  $\mu=0.17$  M ( $\square$ , dashed line). Ratios on the x-axis indicate the molar ratio of negative (DNA) charges to positive (polymer) charges, assuming 100% ionization of each at pH 7.4. The y-axis represents relative turbidity  $(100-T)/(100-T_{\min})$ . Data points represent average  $\pm$  standard deviation ( $n=3$ ). Used with permission from reference [33].

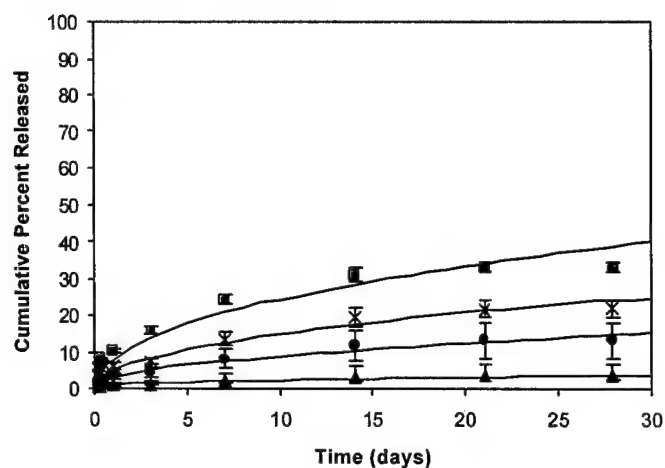


**Figure 3.** *In vitro* bioactivity of *Renilla* luciferase plasmid DNA after encapsulation in SELP-47K hydrogels for various periods of time. DNA encapsulated in the hydrogels retained bioactivity for at least 28 days. Control DNA was used to prepare the hydrogels prior to incubation at 37 °C. Each data point represents the mean  $\pm$  standard deviation for n=3 samples. To be used with permission from reference [56] just submitted.

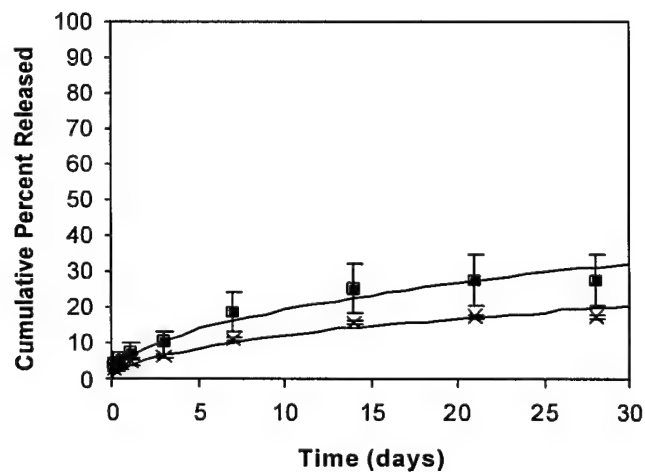


**Figure 4.** Effect of pRL-CMV conformation on release from SELP-47K hydrogels: (●) Linear, (■) Supercoiled, (×) Open-circular; (—) Theoretical release based on an equation for two-dimensional diffusion from a cylinder [61]. Each data point represents the mean  $\pm$  standard deviation for  $n=3$  samples. To be used with permission from reference [56] just submitted.

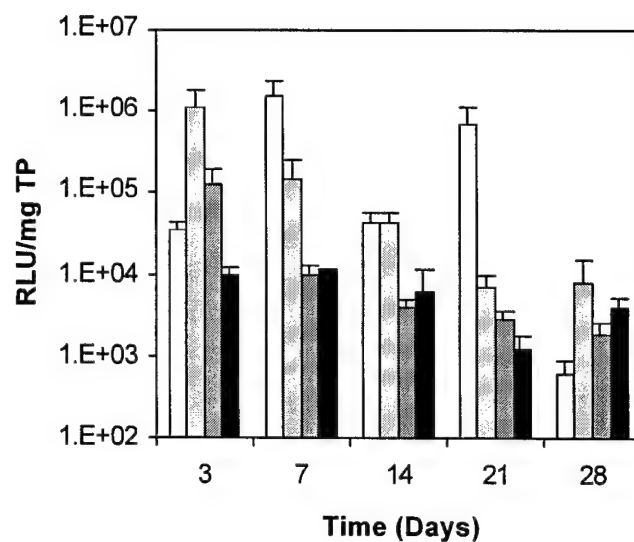




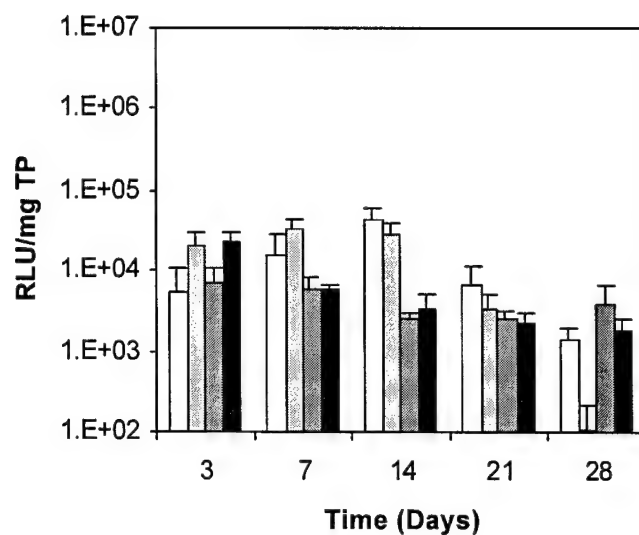
**Figure 5.** Effect of plasmid DNA size on release from SELP-47K hydrogels: (■) pUC 18 [2.6 Kbp], (×) pRL-CMV [4.08 Kbp], (●) pCFB-EGSH-Luc [8.5 Kbp], (▲) pFB-ERV [11 Kbp]; (—) Theoretical release based on an equation for two-dimensional diffusion from a cylinder [61]. Each data point represents the mean  $\pm$  standard deviation for  $n=3$  samples. To be used with permission from reference [56] just submitted.



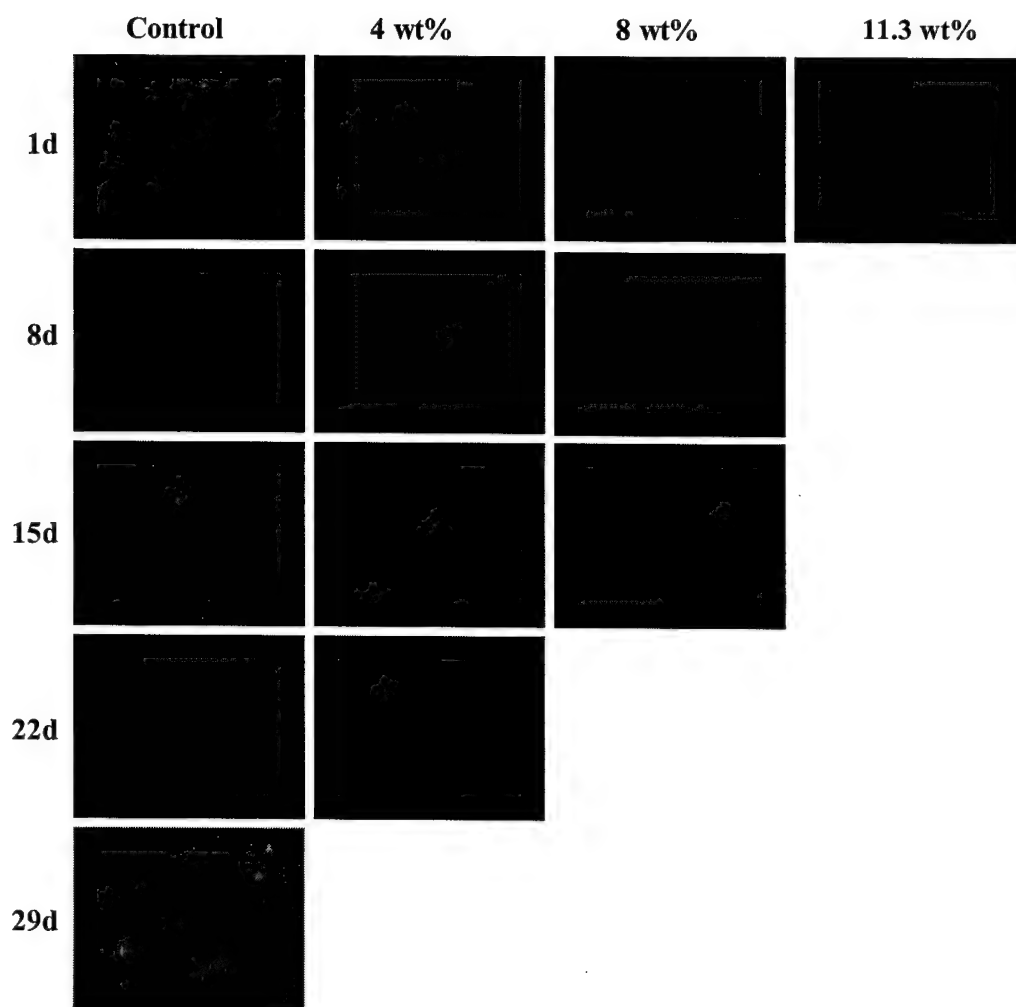
**Figure 6.** Effect of SELP-47K hydrogel dimensions on release of pRL-CMV: (■) disc, (×) cylindrical. (—) Theoretical release based on an equation for two-dimensional diffusion from a cylinder [61]. Each data point represents the mean  $\pm$  standard deviation for  $n=3$  samples. To be used with permission from reference [56] just submitted.



**Figure 7.** Expression of *Renilla* luciferase in MDA-MD-435 tumors grown subcutaneously in athymic *nu/nu* mice, after intratumoral injection. Bars represent 4 wt% polymer (white), 8 wt% polymer (light gray), 12 wt% polymer (dark gray), and naked DNA without polymer (black). Each bar represents the mean  $\pm$  standard error of the mean for  $n=4$  or  $n=5$  samples. To be used with permission from reference [56] just submitted.



**Figure 8.** Expression of *Renilla* luciferase in the skin directly surrounding (~1 cm) MDA-MD-435 tumors grown subcutaneously in athymic *nu/nu* mice. Bars represent 4 wt% polymer (white), 8 wt% polymer (light gray), 12 wt% polymer (dark gray), and naked DNA without polymer (black). Each bar represents the mean  $\pm$  standard error of the mean for n=4 or n=5 samples. To be used with permission from reference [56] just submitted.



**Figure 9.** Adenovirus release from SELP-47K and the corresponding bioactivity results. The percentage of polymer increases from left to right. The time of release of virus from gels used to transfect cells or control increases from top to bottom. The images are from fluorescent microscopy at 40X magnification. Bright spots represent individual cells transfected with AdGFP. To be used with permission from reference [56] just submitted.

## RECOMBINANT POLYMERS FOR DRUG DELIVERY

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## 1. Introduction

Proteins are among the most exquisite components of nature's cellular machinery. Just 20 amino acids comprise the reservoir from which molecules can be constructed that perform such diverse functions as DNA replication, active transport of biological cargo, and structural scaffolding for the cell and organism. However, the amazing versatility of proteins comes at a cost, namely a requirement for exquisite fidelity in their synthesis. The deletion or misplacement of as few as one amino acid can render a protein dysfunctional and have catastrophic consequences for the cell and organism. In order to assure their functionality, elegant mechanisms have evolved for the synthesis of proteins. These include the various regulatory elements and checkpoints that make up the DNA replication and protein synthesis pathways, ensuring that genes are faithfully replicated during cell division, and that proteins with the correct sequences and post-translational modifications are produced from the corresponding genes.

From a polymeric materials science perspective, the properties of biologically synthesized proteins are interesting on three fundamental levels: *functionality*, *diversity*, and *fidelity*. The use of proteins or their subunits in polymeric materials offers the possibility to incorporate biofunctional and/or biorecognizable motifs that can interact with the physiological environment. Examples of biorecognizable materials include those containing recognition sites for proteolytic enzymes for controlled biodegradation, and cellular attachment motifs. As described in the previous paragraph, naturally occurring proteins exhibit tremendous diversity in their material properties and functionality. This diversity can be further enhanced by engineering slight alterations in the sequences of natural proteins, or combining natural proteins to produce artificial

proteins with hybrid functionalities. The silk-elastinlike protein polymers, a hybrid class of materials based on silk fibroin and mammalian elastin, are one example [1]. Finally, the fidelity with which biological systems synthesize proteins offers precise control over sequence, composition, stereochemistry, and molecular weight that is largely unattainable by traditional chemical methods. This fidelity can be exploited to synthesize macromolecules with complex structures and rational placement of functional motifs.

Taken together, all of these characteristics have stimulated significant interest in the use of protein-based biomaterials for drug delivery, gene delivery, and other biomedical applications. The purpose of this chapter is to present the current state of the art in the use of biologically synthesized, protein-based polymers for biomedical applications, with an emphasis on drug and gene delivery.

### *1.1. Genetically Engineered Polymers*

Cappello has defined *genetically engineered protein-based polymers* (hereafter also referred to as *genetically engineered polymers* or *protein-based polymers*) as polypeptide chains composed of tandemly repeated amino acid monomer units, synthesized by recombinant techniques [2]. These monomer units are ligated, at the DNA level, to form a template for the synthesis of a high molecular weight, repetitive polymer. In contrast, *sequential polypeptides* are synthesized by chemical polymerization of short peptides, which are usually obtained by solid-phase techniques. Random *poly (amino acid)* homo- or copolymers are synthesized by random polymerization of a homogeneous or heterogeneous pool of amino acids.



Both sequential polypeptides and poly (amino acid)s have heterogeneous molecular weights. Though sequential polypeptides offer some degree of control over sequence and composition, random poly (amino acid) copolymers have a random sequence and their composition depends on the method of synthesis and reactivity of the comonomers. By contrast genetically engineered polymers have homogeneous molecular weights and recombinant techniques enable long-range and high fidelity control over polymer sequence.

## **2. Synthesis and Characterization**

All strategies for the production of genetically engineered polymers rely on the basic principle of self-ligation (concatamerization) of DNA monomers to form concatameric DNA sequences [3, 4]. These concatamers are then cloned into the appropriate plasmid and introduced into a biological expression system, where they are transcribed and translated by the cellular machinery to produce a protein-based polymer.

Synthesis begins with the conceptual design of the desired polymer and the corresponding oligonucleotide sequence that encodes the monomer(s). When designing these sequences, several biological constraints are considered: First, the codon usage preference of the organism in which the polymers are to be synthesized (often *Escherichia coli*) must be considered. Repetitive usage of rare codons results in transfer RNA (tRNA) depletion, completely inhibiting the synthesis of some polymers and causing truncation of others. Second, while polymeric gene sequences require repetition by nature, the repetition of identical codons should be minimized. This can be achieved by utilizing the redundancy of the genetic code. The consequences of highly repetitive

codon usage include tRNA depletion and genetic instability due to recombination and/or deletion [5-7]. Third, the sequence should be designed to minimize complementarity that can cause messenger RNA (mRNA) secondary structure formation. Formation of mRNA secondary structures can cause pausing and disengagement of the ribosome during translation of the mRNA, resulting in truncated polymers.

The monomer oligonucleotide sequence is typically synthesized by automated chemical synthesizers, which are limited in their ability to produce oligonucleotides with a size greater than 100 bases. This size limitation may require the synthesis of multiple oligonucleotides, which can then be enzymatically ligated to form monomers with a length greater than 100 bases. After synthesis, the monomers are purified and annealed with their complementary strands to make double-stranded DNA that is suitable for cloning.

Synthesis of recombinant proteins (polymers) is often accomplished through the use of two types of plasmids (or vectors), the *cloning* vector and the *expression* vector. Cloning vectors are plasmids that lack the DNA sequences necessary for the transcription of an inserted gene. They are normally used to "store" genes in a stable form until expression and to limit the problems that can arise from basal levels of expression of toxic proteins. Expression vectors contain the DNA sequences necessary for gene transcription and, in combination with an appropriate biological host, can be used to produce a protein (polymer) of interest. To minimize genetic instability early in the process, a cloning vector is often used in the initial stages of polymer synthesis. The monomer gene is inserted into such vector and transformed into *E. coli*, which can be grown to produce large quantities of the vector and hence the monomer gene within it.

Before multimerization, the presence and identity of the monomer gene are confirmed by restriction digestion and DNA sequencing [8].

In order to produce polymers, at the DNA level, the monomers must be concatamerized. The first step in the synthesis of concatamers is the production of relatively large amounts of the monomer. This can be accomplished through large-scale plasmid preparations or the polymerase chain reaction (PCR), followed by digestion with the appropriate restriction enzymes and gel purification. The classic technique for concatamerization of DNA monomers involves the incubation of monomers with ligation enzymes such as T4 DNA ligase [1]. This strategy produces an assortment of DNA concatamers in which the size may be roughly controlled by the reaction time and the concentration of the monomers and ligase. However, this technique suffers from three primary limitations: First, there is no guarantee that a concatamer of a desired size will be obtained. Second, as concatamer size increases, circularization occurs, preventing further manipulation. Third, this method requires the inclusion of unique restriction enzyme recognition sites that may require the insertion of extraneous codons (and hence amino acids) between monomers. These limitations have been addressed by the development of altered synthetic strategies. Recursive directional ligation is a method that can be used to synthesize concatameric DNA sequences of precisely defined length while avoiding circularization problems [9]. Another technique, relying on the isolation and ligation of pre-ligated concatamers has also been utilized to control length and limit circularization [10]. Finally, a technique called *seamless cloning* has been used to produce DNA monomers and concatamers without any extraneous codons between monomers [11, 12].

After synthesis, the DNA concatamers are ligated into either another cloning vector or an expression vector. The choice of the vector is partially based upon the availability of the necessary restriction sites and desired purification strategy. When the polymer gene is cloned into an expression vector, it may be transformed first into a strain of bacteria that is incapable of expressing it. As with the use of a cloning vector, this approach avoids potential problems related to genetic stability and/or cytotoxicity due to basal levels of polymer expression. However, the production of polymers eventually requires that an expression plasmid containing the polymer gene be transformed into an expression host.

Many *E. coli* expression systems contain an inducible promoter that can be used to turn on polymer production once the cells have grown to an optimal density. This strategy is intended to maximize the efficiency of polymer production. Production of recombinant proteins redirects metabolic resources that are normally used for cellular growth, maintenance, and division, often leading to a slowing or arrest of growth. By allowing the cells to reach a relatively high density prior to inducing polymer expression, the need for further division can be somewhat circumvented and the yield of the polymer may be increased.

Purification of polymers is performed by standard techniques that have previously been established for recombinant proteins. Frequently these include the use of affinity tags (e.g., poly (histidine), glutathione-s-transferase) that can be used to chromatographically purify recombinant proteins. If necessary, the tag can subsequently be removed by enzymatic cleavage.

Some polymers have been purified on the basis of their physicochemical properties. For example, silk-like polymers have been purified by taking advantage of their low solubility in aqueous medium [13]. Elastin-like polymers (ELPs) have been purified by temperature cycling above and below their inverse temperature transition ( $T_i$ ) [14]. This technique has been extended to produce an ELP-tag that can be used to purify a number of recombinant proteins by temperature cycling, which may be faster and less expensive than affinity chromatography [15].

After purification, polymers are typically characterized by a standard set of techniques that can include amino acid content analysis, mass spectrometry, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting. These methods are intended to verify the identity of the polymer. Depending on the type of polymer being synthesized, a series of polymer-specific characterizations will then be performed.

While biological synthesis of polymers confers many advantages, it also imposes some limitations. As described previously, there are several issues that must be considered when designing the monomer and concatamer gene sequences. Furthermore, the potential toxicity of the genetically engineered polymer to the expression host, and the resultant effect on the integrity of the product must be considered.

While the 20 natural amino acids do provide significant structural diversity, the natural amino acid pool can also be viewed as a limitation. Kiick *et al.* have begun to address this limitation by incorporating artificial amino acid analogs into genetically engineered polymers [16]. Incorporation of the amino acid azidohomoalanine into the medium of methionine-depleted bacterial cultures resulted in the replacement of natural

methionine with azidohomoalanine. The significance of this substitution is that it allows chemical modification of the azide group by the Staudinger ligation, offering the ability for site-specific modification of the protein-based polymer.

### **3. Drug Delivery Applications**

Drug delivery research is substantially focused on improving methods to deliver medications to the necessary location, in the correct amount, at the correct time. The relatively recent emergence of nucleic acid based therapies has enhanced the appreciation for protection and targeting at the subcellular level. Because of the potential to incorporate several functions into a single molecule, there is significant interest in polymeric biomaterials for drug and gene delivery applications requiring the circumvention of multiple biological barriers. The ability of some polymers to transverse these barriers and deliver bioactive agents without significant toxicity lies primarily in their chemical structure. By enabling more precise control over the macromolecular architecture, the structural factors influencing the effectiveness of drug delivery from polymeric biomaterials can be more clearly elucidated, and more efficient delivery systems can be designed.

Though still at an embryonic stage of development, the application of genetically engineered polymers to drug delivery thus far can be divided into two general approaches: polymers for systemic administration and gel-forming polymers for localized, controlled release applications. Both approaches may yield either a localized (i.e., targeted) or systemic effect, depending on the macromolecular structure and the properties of the therapeutic entity.

### *3.1. Polymers for Systemic Drug Delivery*

#### *3.1.1. Thermally Targeted Elastin-Like Polymeric Carriers*

Applications of genetically engineered polymers for targeted systemic drug delivery have primarily aimed at treating solid tumors. The targeting of anticancer medications to solid tumors is an extremely challenging problem, due to the vascular and structural heterogeneity, and elevated hydrostatic pressure within the tumor. Traditional methods of tumor targeting have relied on affinity approaches, selectively targeting specific epitopes on tumor cells, or the passive enhanced permeability and retention (EPR) effect. EPR refers to the enhanced permeability of the tumor vasculature and retention of fluids within the tumor, due to a locally dysfunctional lymphatic drainage system [17]. Macromolecular carriers, of appropriate molecular weight, passively accumulate within tumors by transport across the leaky tumor vasculature. One approach to increase the efficiency of this accumulation involves using localized hyperthermia to induce aggregation of thermally responsive polymeric carriers within the tumor.

Elastin-like polymers (ELPs) are macromolecules composed of the monomeric pentapeptide (VPGXG), where X can be any amino acid residue except proline [18, 19]. Through variation in the length of the polymer and the residue in the X position, numerous polymers have been synthesized that exhibit sharp phase transitions, due to hydrophobic collapse and aggregation, when the temperature is raised above a temperature termed the inverse temperature transition ( $T_i$ ) [19]. The responsiveness of these materials to temperature can be further modulated by combining monomers with

different X residues, to construct block copolymers in which the constituent blocks undergo phase transitions at distinct temperatures [20, 21].

Chilkoti and coworkers have pioneered the use of ELPs as thermally targeted carriers for the treatment of solid tumors [22]. Using genetic engineering techniques, they constructed a library of ELPs with various sequences and molecular weights, both of which influence  $T_t$ . From this library, a polymer was selected with a  $T_t$  of approximately 40 °C, intermediate between body temperature ( $\approx 37$  °C) and a temperature induced by localized heating of the tumor ( $\approx 42$ -43 °C). This approach enhances localization, by adding thermally induced aggregation of the ELP within the tumor. Furthermore, localized heating of tumors is known to increase macromolecular extravasation and sensitivity to some therapeutics [23, 24].

Prior to *in vivo* characterization of the thermally targeted ELP, *in vitro* characterizations were performed to evaluate the effects of drug conjugation, solvent, and polymer concentration on  $T_t$  [22]. Conjugation of reporter molecules to the ELPs was achieved by chemical coupling to a short N-terminus peptide leader sequence (e.g., SKGPG), engineered at the genetic level. Conjugation of the reporter molecules iodobenzoate and rhodamine reduced the  $T_t$  of the ELPs, possibly due to increased hydrophobic interactions between the polymers. Analysis of the phase transition in murine and mock (PBS + 0.9 mM BSA) plasma was found to decrease  $T_t$  by approximately 4°C. Although increasing the concentration of polymer resulted in a decrease in  $T_t$ , the  $T_t$  of the selected polymer remained within the desired temperature range (37-42 °C) over an approximately 10-fold range of concentration.



Intravenous administration of the thermally responsive ELP-rhodamine conjugate resulted in localized precipitation in tumor tissue heated to 42 °C, while no precipitation was observed when a control ELP-rhodamine conjugate, with a  $T_t$  of 70 °C, was administered. Localized hyperthermia was observed to result in a two-fold increase in intratumoral accumulation and a two- to three-fold increase in cellular uptake when compared to unheated controls [22, 25, 26]. ELP-doxorubicin conjugates induced cytotoxicity comparable to free doxorubicin *in vitro*, despite differences in the subcellular localization of the free and conjugated drug [27]. Free doxorubicin was observed to accumulate in the nucleus, while the ELP-doxorubicin conjugates were dispersed throughout the cytoplasm, with significantly less nuclear accumulation, indicating the possibility of a difference in the mechanism of action of the two forms. These studies highlight the potential of temperature-responsive genetically engineered polymers as carriers for anticancer medications and have been reviewed in detail elsewhere [21, 28].

### 3.1.2. Micelle-Forming Polymers

As mentioned previously, ELPs can be further customized by combining different pentapeptide blocks, to produce block copolymers with sensitivity to several different temperatures [20, 21]. Block copolymers are of great interest for drug and gene delivery applications, significantly because of their ability to form micellar structures with the ability to encapsulate drugs or nucleic acids within the core, and to display targeting moieties on the periphery of the shell [29].

Conticello and colleagues have intensively studied the potential of amphiphilic diblock (**AB**) and triblock (**ABA**) elastin-like copolymers, where **A** is a hydrophilic and **B**

a hydrophobic block, to reversibly self-assemble into well-defined micellar aggregates [20, 30]. Collapse of the hydrophobic block above  $T_t$  results in the formation of elastin-based nanoparticles. To provide diversity in the mechanical properties of the micellar structures, the amino acid sequence of the hydrophobic block was varied between plastic (VPAVG) and elastomeric (VPGVG) in nature. The hydrophilic block is designed to maintain solubility and form a protective core that prevents protein adsorption and clearance by the reticuloendothelial system.

Reversible self-assembly into monodisperse spherical micellar particles, 50-90 nm in diameter, was observed for diblock copolymers (**AB**) and triblock copolymers (**ABA**) [30]. The temperature-dependent loading of solutes into these micelles was demonstrated with the fluorescent probe 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS) [31]. The emission intensity of the fluorophore was observed to undergo a sharp change at 15 °C, which coincided with the phase transition temperature of the hydrophobic block, indicating that the probe was encapsulated within the hydrophobic core of the micelle.

Chilkoti and coworkers have synthesized elastin-like block copolymers composed of one block with a  $T_t$  of 35 °C and another block with  $T_t$  of >90 °C [9]. This block copolymer was designed such that the block with the lower  $T_t$  should collapse and aggregate above 35 °C, while that with the higher  $T_t$  should remain solvated below 90 °C, thus forming a core-shell structure. The evolution of particle formation was followed, by dynamic light scattering (DLS) and turbidimetry, as a function of temperature [9]. The results from both techniques indicated that the particle size of the elastin-like block copolymer solutions changed in four distinct steps as the temperature increased. The structures formed during each phase were hypothesized to be free elastin-like block

copolymer (4.4 nm, ~35-40 °C), micellar nanoparticle (20.4 nm, ~40-47.5 °C), rearranged nanoparticle (54.5 nm, 47.5-50.8 °C), and finally aggregate (1,400 nm, >50.8 °C). This work demonstrates the potential to precisely control nanoparticle size, and potentially drug loading and unloading, through variations in temperature. Biological fate of polymeric micelles, such as vascular extravasation and cellular uptake are influenced by the particle size.

### *3.2. Hydrogel-Forming Polymers*

Hydrogels are water-swollen polymer networks formed by chemical or physical crosslinking of the polymeric chains. Chemical crosslinking involves the formation of covalent bonds between crosslinking reagents and corresponding functional groups on the polymer chains. Physically crosslinked hydrogels can form by a number of mechanisms, including ionic interactions, crystallization, and hydrophobic interactions. For a thorough review of hydrogel crosslinking methods, the reader is referred to a recent comprehensive article by Hennink and van Nostrum [32].

Perhaps due to the fact that hydrogels can absorb large quantities of water, they tend to be relatively biocompatible. This biocompatibility, as well as the ability to control the swelling and deswelling by varying the crosslinking density, and the incorporation of stimuli-responsive elements, has made hydrogels the subject of intensive study in the controlled drug delivery field. The release of solutes from hydrogels is governed by the physicochemical properties of the polymer, the degree of crosslinking, and the properties of the solute. Recent developments in hydrogels and their biomedical applications including controlled release have been reviewed in reference [33].

### *3.2.1. Thermoreversible Hydrogels from Elastin-Like Polymers*

In addition to the characterizations of the micelle-forming diblock and triblock copolymers, previously described, Conticello and coworkers have also synthesized and characterized triblock copolymers in which the hydrophobic blocks constitute the end units (**BAB**) [34]. These copolymers undergo hydrophobic self-assembly to form physically crosslinked, thermoreversible hydrogels when the temperature is raised above the  $T_t$  of the hydrophobic **B** block. The polymers could be molded into various shapes by injecting them in the solution state and then incubating the mold above  $T_t$  [20]. Scanning electron microscopy of flash-frozen gels showed networks of micellar aggregates, with individual micelles ranging from 20-30 nm in diameter. The complete reversibility of the sol to gel transition in these hydrogels is notable and distinct from hydrogels formed by chemical crosslinking of ELPs.

### *3.2.2. Silk-Elastinlike Hydrogels*

The silk-elastinlike family of protein block copolymers (SELPs) are one class of genetically engineered biomaterial that has received considerable attention for applications in drug and gene delivery. These polymers are composed of tandemly repeated units of silk-like (GAGAGS) and elastin-like (GVGVP) peptide blocks [35]. X-ray diffraction studies have shown that the silk-like blocks self-assemble via hydrogen bonding to form  $\beta$ -sheet crystals that impart thermal and chemical stability [36, 37]. Periodic inclusion of elastin-like blocks decreases the overall crystallinity of the material and increases its aqueous solubility [2]. The biological, physicochemical, and material properties of the polymer chains can be specifically tailored, by varying the block

lengths, sequence, and compositional ratio [2]. For an in-depth review of the properties of silk-elastinlike polymers, the reader is referred elsewhere [38].

#### *3.2.2.1. Gelation of Silk-Elastinlike Polymers*

Selected polymers from the SELP family (e.g., SELP-47K, Figure 1) spontaneously and irreversibly form hydrogels under physiological conditions, and in the absence of solvents, crosslinking reagents, or reactive monomers [39]. The kinetics of the sol to gel transition are dependent on the solution conditions and the structure and concentration of the polymer. Bioactive molecules can be incorporated homogeneously into the hydrogel matrix by simple mixing with the liquid polymer solution, prior to gelation [39-41].

The formation of SELP hydrogels has been characterized by differential scanning calorimetry (DSC) and rotational viscometry [39]. Consistent with x-ray diffraction results, DSC revealed an exothermic peak that indicates self-assembly via crystallization of the silk-like blocks. The rate of crystallization, and thus gelation, was enhanced by the addition of small quantities of the appropriate seed crystals and disrupted by the addition of 6M urea, which disrupts hydrogen bonds. The rate of increase in the viscosity of SELP solutions was directly related to the number of silk-like blocks contained in the polymer. SELP-0K, containing two silk-like blocks, exhibited no increase in viscosity over the 125 minute analysis, while SELP-5 and SELP-47K both exhibited significant increases in viscosity. SELP-5, containing eight silk-like blocks, was observed to gel approximately twice as fast as SELP-47K, with four silk-like blocks. This difference was attributed to a lag phase of approximately one hour for SELP-47K, over-which no

increase in viscosity was observed. Given enough time (i.e., twice as much) for gelation, SELP-47K attained a viscosity equal to that of SELP-5. A positive correlation was observed between temperature and gelation rate, though SELP-5 gelled faster than SELP-47K at all temperatures.

#### *3.2.2.2. Swelling of Silk-Elastinlike Hydrogels*

The degree of swelling describes the amount of water imbibed by a hydrogel network. This parameter provides insight into the relative crosslinking density of the network and hence its porosity and solute transport characteristics. Swelling studies showed that physically crosslinked SELP-47K hydrogels were relatively insensitive to changes in pH and ionic strength [42]. This is in contrast to chemically cross-linked elastin-like hydrogels without silk-like blocks [43]. This insensitivity was explained by the irreversible crystallization of the silk-like blocks. However, decreasing the polymer concentration from 10 to 8 wt% resulted in an increase of over 50% in the degree of swelling. A significant decrease in swelling was observed as the cure time increased from 1-hour to 24-hours. At reduced concentrations or cure times there is a lower probability that the polymer chains will interact, therefore decreasing the cross-linking density, which in turn leads to a higher degree of swelling. These results indicate that the swelling ratio of the SELP hydrogels studied is influenced more by polymer concentration and cure time, than by changes in environmental stimuli such as temperature, pH, and ionic strength.

Increasing the concentration and / or cure time of the hydrogels also resulted in less polymer soluble fraction [42]. In addition, the sample-to-sample variability in

soluble fraction decreased at the longer cure time (24-hours). As with decreased swelling, these results were also attributed to an increased density of physical cross-links with increasing polymer concentration and cure time.

The soluble fraction data were used to calculate equilibrium polymer concentrations and equilibrium hydration of the hydrogels [42]. The data indicate that, after release of the initial soluble fraction, no hydrogel degradation or dissolution occurred.

#### 3.2.2.3. Drug Delivery from Silk-Elastinlike Hydrogels

The ability of SELPs to form hydrogels *in situ*, along with their biocompatibility and customizable structure, has stimulated interest in their use for the localized, controlled delivery of therapeutic agents. To evaluate the suitability of SELP hydrogels as controlled delivery vehicles, Cappello *et al.* studied the release of several fluorescently labeled probes, with molecular weights ranging from 380 to 70,000 Da [39]. The release kinetics of these probes were all approximately first-order, with a negative correlation between molecular weight and release rate. Interestingly, no significant difference in the time for release of 50% of the loaded compound ( $T_{50}$ ) was observed between dansyl-lysine (MW=380) and fluorescein-dextran (MW=10,000). This indicates a relatively large pore size in these hydrogel networks, which do not significantly restrict the diffusion of solutes in this molecular weight range.

The delivery of proteins from SELP hydrogels was first investigated by Cappello and coworkers, who studied the release of a recombinant mitotoxin (Pantarin) from SELP-47K hydrogels [39]. This protein inhibits the proliferation of cells expressing the

FGF receptor, making it an attractive candidate for localized delivery to tumors. Release of bioactive  $^{125}\text{I}$ -labeled protein occurred over a period of eight days, with a significant burst effect in the first 24-hours.

The delivery of another protein, cytochrome c (MW=12,384) and two smaller compounds vitamin B<sub>12</sub> (MW=1,355) and theophylline (MW=180) were investigated by Dinerman *et al* [41]. Release curves of equilibrium-loaded hydrogels fit a mathematical model for two-dimensional diffusion from a cylinder, in the axial and radial directions, indicating the occurrence of Fickian diffusion (Figure 2) [44, 45]. Increasing the hydrogel cure time from 1 hour to 24 hours reduced the intra-gel diffusivity of theophylline and vitamin B<sub>12</sub>, but not cytochrome c, which remained essentially constant despite a decrease in swelling with longer cure time. Calculation of normalized solute diffusivities indicated hindered transport in all three cases.

To quantify the influence of partitioning on solute transport, normalized diffusivity and dimensionless permeability were compared for each solute [41]. Increasing the cure time of the hydrogel from 1 hour to 24 hours decreased the partition coefficient for all three compounds, though this decrease was not statistically significant for theophylline, indicating potentially size-dependent partitioning behavior [41]. With a 1 hour cure time, only the transport of vitamin B<sub>12</sub> appeared to be affected by partitioning. In hydrogels cured for 24 hours, solute partitioning was found to significantly affect the transport of both vitamin B<sub>12</sub> and cytochrome c. The polymer volume fraction influenced both the effective diffusivity and partitioning of cytochrome c, though this effect was minimal in the volume fraction range of 0.04 to 0.09. A more pronounced effect was observed at greater polymer volume fractions (>0.1).



Direct incorporation of cytochrome c into 12 wt% SELP-47K solutions prior to gelation yielded hydrogels that released nearly all of the loaded dose within two hours [41]. Cure time did not influence the effective diffusivity of cytochrome c over a range of 1 to 24 hours, despite a decrease in the degree of hydration. Equilibrium uptake loading of pre-formed hydrogels with cytochrome c yielded effective diffusivities that were similar to those obtained by the direct incorporation method, indicating that either technique may be used to load the hydrogels.

The equilibrium swelling ratio and gel dimensions were determined before and after release, for the hydrogels in which cytochrome c was directly incorporated [41]. Regardless of gelation time, release of cytochrome c was accompanied by an approximately two-fold increase in hydrogel swelling. Despite the change in swelling, the dimensions of the gels remained essentially constant, suggesting that the increased swelling is probably due to a decreased cross-linking density in the hydrogel network, resulting in the release of soluble fraction throughout the study.

### *3.2.3. Hybrid Hydrogels*

Hybrid polymers made from genetically engineered motifs and chemically synthesized constructs have been made with the intent of expanding the diversity of materials that are accessible via only the chemical or biological route. Thus far, research has focused substantially on the incorporation of protein subunits, with well-defined physicomachanical properties, into chemically synthesized polymers. The overall mechanical properties of proteins are determined by the mechanical properties of the individual, modular components. These modular “building blocks” are present in a

number of proteins and underlie many important physiological processes. One example is the fibronectin type III (Fn3) module, found in proteins that exhibit responsiveness to mechanical force. In the giant muscle protein titin, the unfolding of Fn3 and immunoglobulin-like (Ig) modules allows the protein to alter its spring constant in response to force [46]. These structural protein modules can serve as a resource for the development of new biomaterials with well-defined properties and unique responses to environmental stimuli.

Chen, Kopecek, and Stewart have utilized the Ig domain of titin to cross-link water-soluble N-(2-hydroxypropyl)methacrylamide copolymers [47]. The hydrogels were formed by chelation between a 6X histidine tag, engineered on the ends of the Ig domain, with  $\text{Ni}^{2+}$ -chelated acrylamide copolymer. This approach should be widely applicable to a number of protein crosslinking domains, and allows crosslinking to occur in aqueous medium, at neutral pH, at ambient temperature, and in the absence of free radical generating reagents, thus preserving the structural integrity of the protein. These hybrid hydrogels swell substantially in response to increases in temperature, as a result of the thermally-induced unfolding of the Ig domain. This positive temperature-volume response is unusual in thermosensitive hydrogels and highlights one potentially useful contribution of protein engineering to the biomaterials field.

In addition to the hybrid hydrogels crosslinked with Ig domains, Wang, Kopecek, and Stewart have utilized proteins forming coiled-coils as crosslinking reagents for HPMA hydrogels [48]. Coiled-coils are composed of  $\alpha$ -helices that wrap around each other, forming a left- or right-handed superhelical bundle [49]. The coiled-coil motif is found widely in nature and known to occur in over 200 proteins. Increasing the

temperature of the coiled-coil results in collapse due to unfolding of the rod-like structure.

Hydrogels were synthesized with crosslinkers that varied in the number of coiled-coil repeats (1 to 3 repeats) [48]. Equilibrium swelling of the hydrogels was found to increase as the number of coiled-coil repeats, and hence crosslinker length, increased. On the other hand, increasing the crosslinking density led to a decrease in equilibrium swelling. All hydrogels underwent a thermally induced collapse with increased temperature, with the crosslinkers containing one and three coiled-coil repeats exhibiting a greater change in swelling than those containing two repeats. The temperature at which this transition occurred corresponded well with the known temperature at which the coiled-coil domains are known to unfold. This work highlights the potential of protein polymers in engineering hydrogels that undergo volume transitions at very specific temperatures.

#### **4. Gene Delivery Applications**

It is widely recognized that the current limitations of gene delivery form a bottle neck that prevent safe and effective gene therapy. The major challenges in gene delivery, like drug delivery, can be distilled fundamentally to *spatio-temporal control* over release. Furthermore, since the delivery of nucleic acids may result in short or long-term effects, a method by which the expression of the delivered gene can be regulated would be desirable.

We have investigated the potential of genetically engineered SELP-47K to act as an *in situ* gel-forming matrix for the controlled, long-term delivery of plasmid DNA and

adenoviral vectors to solid tumors [40, 50]. The use of polymeric matrices for gene delivery allows manipulation of the release profile of the gene, potential protection against degradative enzymes such as nucleases and proteases, and precise spatial localization at the desired site of action.

The current limitations of actively targeted, systemic cancer gene delivery systems make intratumoral injection an attractive alternative. The primary advantages of intratumoral gene delivery stem from its very precise spatial localization. Direct injection into the tumor obviates the need for systemic targeting, though the efficiency of the therapy may be increased by attaching tumor-specific ligands to the vector [51]. The primary disadvantage of intratumoral gene therapy is that, unless a significant bystander effect occurs, each tumor must be individually identified and injected. Hence, by virtue of the difficulty in their detection and distributed nature, small metastases could be impossible to treat by this method. Intratumoral cancer gene therapy may thus be best suited for the treatment of cancers that grow slowly (e.g., prostate cancer) and/or where potential complications or disability make tumor resection less desirable (e.g., prostate cancer, head and neck cancer).

#### *4.1. Delivery of Plasmid DNA from Silk-Elastinlike Hydrogels*

Our objective is to design gene delivery systems that can deliver plasmid DNA and viral vectors over a period of at least 28 days, thus ensuring that the expression of the transgene occurs for therapeutically relevant periods of time. As a first step toward this goal, the *in vitro* release of plasmid DNA from SELP-47K hydrogels has been evaluated over a 28 day period [40, 50]. DNA-containing hydrogels were fabricated by mixing

aqueous DNA solution with aqueous SELP-47K solution. DNA/polymer solutions were incubated at 37 °C, for 1 or 4 hours to induce gelation

DNA release was initially evaluated as a function of polymer concentration, DNA concentration, ionic strength, and cure time [40]. Consistent with previous swelling studies, increasing the polymer concentration and/or cure time decreased the rate of DNA release from the hydrogels. Within a range of 50 or 250 µg/ml, the DNA concentration did not influence the rate of release. DNA release was strongly affected by the ionic strength of the medium, due to an ionic interaction between the negatively charged DNA and positively charged SELP (Figure 3). Turbidimetric analysis confirmed an interaction between the polymer and DNA that was dependent on the ionic strength of the medium and consistent with the release data. Such ionic interactions between macromolecular solutes and hydrogel networks have previously been used to control solute release. Genetic engineering techniques offer the ability to precisely modulate the charge density and periodicity of the network, thereby facilitating the study of these interactions with a fidelity that is difficult to achieve by chemical methods alone.

DNA release was observed for greater than 28 days, with apparent intra-gel diffusivities of approximately  $10^{-9}$  to  $10^{-10}$  cm<sup>2</sup>/s, depending on the polymer concentration and cure time [40]. Transfection studies performed on encapsulated DNA indicated that it retained *in vitro* bioactivity equivalent to stock DNA for at least 28 days [50].

The conformation of plasmid DNA, namely supercoiled, open circular, or linear has been hypothesized to play a role in its transfection efficiency. While conventional thinking has generally indicated a preference for supercoiled DNA, at least one study has shown that the delivery of linear DNA results in prolonged transgene expression *in vivo*,

due to concatamerization [52]. Other studies have shown that the conformation of DNA is irrelevant to the transfection efficiency *in vitro* and *in vivo* [53].

In order to evaluate the influence of plasmid conformation on release from SELP-47K hydrogels, plasmid DNA predominantly in the supercoiled, open circular, and linear conformations were prepared [50]. The linear form of plasmid DNA was released most rapidly from the hydrogels, followed by the supercoiled form (Figure 4). The open circular form was practically not released, probably due to its impalement on the polymer chains. The influence of plasmid size on release was also investigated, with size-dependent release observed for plasmids from 2.6 to 11 kilobases (kb) in size, from 10 wt% SELP hydrogels [50]. Since hydrogels form from SELPs with concentrations as low as 4 wt%, it is possible that plasmids larger than 11 kb could be delivered.

The effect of hydrogel geometry on DNA release was also studied by fabricating hydrogels in the form of cylinders and flat discs [50]. Disc-like hydrogels released DNA faster than their cylindrical counterparts. This was attributed to their larger surface to volume ratio and was accurately described by fitting the release data to an equation that describes two-dimensional diffusion from a cylinder with geometric considerations [44, 45].

While the applications of SELP-mediated controlled gene delivery are numerous, we have focused our efforts on controlled delivery to solid tumors. Our initial studies in this arena involved the intratumoral delivery of a reporter plasmid (*Renilla* luciferase) to solid tumors in a murine (athymic *nu/nu*) model of human breast cancer (MDA-MB-435 cell line) [50]. Delivery of the *Renilla* luciferase plasmid from SELP-47K matrices resulted in significantly enhanced tumor transfection for up to 21 days when compared to

naked DNA (Figure 5). In particular, delivery of the plasmid from matrices containing 4 or 8 wt% polymer resulted in enhanced transfection up to 21 days, while 12 wt% matrices enhanced transfection up to 3 days. These results are consistent with sustained delivery from the 4 and 8 wt% matrices and entrapment of the DNA within the 12 wt% matrix.

The levels of tumor transfection mediated by the three concentrations of polymer were statistically equivalent until 7 days, when the 4 and 8 wt% matrices were both more effective than 12 wt%. The greater transfection persisted until 21 days for 4 wt% polymer and 14 days for 8 wt% polymer. Overall, the delivery of DNA from 4, 8, and 12 wt% hydrogels resulted in a mean 142.4-fold, 28.7-fold, and 3.5-fold increase in tumor transfection, respectively, compared with naked DNA over the entire 28 day period.

In addition to the delivery of DNA that occurs within the tumor, it can be expected that some DNA will diffuse from the matrix into to the surrounding tissue. In order to evaluate this, transfection of the skin approximately 1 cm around the tumor was evaluated [50]. The enhancement of delivery to the tumor was compared to the levels of transfection in the tumors and skin at each time point and polymer concentration. While statistically significant differences were not detected between all compositions, the mean tumor transfection was 42.0, 27.2, and 4.6 times greater than skin transfection for 4, 8, and 12 wt% hydrogels, respectively, over the entire 28 day period. This is in contrast to a 1.3 fold difference between tumor and skin transfection for naked DNA.

#### *4.2. Delivery of Adenoviral Vectors from Silk-Elastinlike Hydrogels*

Despite the promising results obtained from delivering plasmid DNA from SELP hydrogels, the delivery of naked DNA results in relatively low transfection efficiency and

thus has limited applications. For this reason, we sought to explore the potential of SELP-47K to act as a matrix for the controlled delivery of viral vectors [50]. The use of viruses that integrate into the host genome (e.g., retrovirus) can ensure long-term expression of a transgene. However, as was recently observed in a study of x-linked severe combined immunodeficiency, insertion of the viral DNA at or near an oncogenic regulation site can lead to the development of cancer [54]. Thus, there is also an intense interest in non-integrating viral vectors, such as adenovirus, which may have a higher margin of safety than integrating viruses. However, without integration into the host genome the duration of transgene expression is limited. A controlled release approach may extend the duration of transgene expression by continuously delivering adenovirus at the site of action. In addition, it is conceivable that the immune response to the virus may be modulated by encapsulation in a polymeric matrix and allowing the release of very small quantities of virus in a given time. Current polymers have not provided the capability to deliver viable adenoviral vectors effectively over prolonged periods of time. Coacervate microspheres of gelatin and alginate have shown poor encapsulation efficiency, low virus bioactivity, and poor virus release kinetics [55]. Collagen-based matrices have potential, but control over the rate of release is complicated by the limited control over polymeric structure and crosslinking density [56, 57]. Genetic engineering techniques may allow the design and synthesis of new polymers with precisely defined architecture and crosslinking density, where biodegradation can be controlled to release viable viral vectors at specific sites and rates.

As a first step toward that goal, we evaluated the potential of SELP-47K hydrogels to act as matrices for the controlled delivery of an adenovirus containing the



green fluorescent protein (gfp) reporter gene (AdGFP) [50]. SELP-47K/AdGFP solutions were prepared at 4, 8, and 11.3 wt% polymer, allowed to gel, and placed in a PBS release medium. At predetermined time points, release medium was collected, entirely replaced, and used to transfect HEK-293 cells. These cells contain a relatively high density of adenoviral receptors and are thus a good screening tool for the presence and bioactivity of adenovirus in the release medium. Transduction was observed up to 22 days with the viruses released from the 4 wt% hydrogel (Figure 6). The number of transduced cells obtained with the viruses released from the 8 wt% hydrogel was fewer than that obtained from the 4 wt% hydrogel. The 11.3 wt% hydrogel did not release any detectable adenovirus after the first day. These experiments demonstrate that adenoviral release can be controlled over a continuum by varying polymer composition from no release (11.3 wt% gel) to greater release (4 wt% gel). Control samples (viral particles without hydrogels in release medium, were bioactive until day 29 (with few gfp+ cells on day 29). However, as anticipated, bioactivity of the vectors decreased over time. Future research will include quantifying the amount of adenovirus released, the proportion that is bioactive, and the *in vivo* biodistribution, efficacy, and toxicity of adenoviral particles delivered from SELP-47K.

#### 4.3. Soluble Nucleic Acid Carriers

A genetically engineered silk-like polymer derivatized with cationic functional groups, Pronectin F+, has recently been studied as a nonviral vector for gene delivery [58]. Pronectin F is a silk-like polymer with one fibronectin domain between every nine silk-like repeats. This polymer was originally synthesized by Cappello and colleagues,

and has been explored as a substrate to enhance cellular attachment to hydrophobic materials [35].

Hosseinkhani and Tabata cationized Pronectin F by reaction of ethylenediamine, spermidine, and spermine with the hydroxyl groups of the serine residue in the silk-like blocks (GAGAGS). For comparison, similarly cationized derivatives of gelatin were prepared. When complexed with plasmid DNA at a weight ratio of 50:1 protein:DNA, all three cationized Pronectins (Pronectin F+) formed particles with DNA with slightly positive ( $\sim 10$  mV) zeta potential and a size of approximately 200 nm.

All three Pronectin F+ derivatives increased the *in vitro* transfection of rat gastric mucosal cells by a reporter (luciferase) plasmid in comparison to naked plasmid. Due to its higher buffering capacity, the spermine derivative was found to be significantly more effective than the ethylenediamine and spermidine derivatives. Cellular attachment mediated by the Pronectin F+ derivatives, containing 13 RGD motifs, was found to be significantly greater than the cationized gelatin, which contained only one RGD motif. The amount of plasmid uptake mediated by the Pronectin F+ derivatives was found to be greater than that mediated by cationized gelatin. This study shows the potential of biorecognizable genetically engineered polymers as soluble nonviral vectors and surface coatings for gene delivery.

## **5. Tissue Repair Applications**

There is significant interest in the synthesis of novel biomaterials for tissue repair or tissue engineering. Many of these materials are designed to include cellular recognition sequences (e.g., RGD) for the attachment of cells at specific sites. Genetic

engineering techniques provide the ability to incorporate these biorecognition sequences at precise locations within the polymer backbone. Furthermore, protease recognition sites can be engineered into the polymer to provide controlled degradation in response to exposure to specific enzymes.

As previously mentioned, Cappello and coworkers have synthesized silk-like polymers that contain periodically spaced fibronectin sequences [35, 59]. These polymers, called SLP-F (also, Pronectin F), have been shown to support the attachment of a variety of cell types, including epithelial cells, fibroblasts, and cancer cells [59]. In addition, silk-like polymers containing regions of human laminan have been synthesized (SLP-L) [2]. The SLP-L polymers promoted greater attachment and spreading of fibrosarcoma and rhabdomyosarcoma cell lines than polylysine or laminan coatings. The inclusion of these attachment sequences in-between silk-like blocks allows them to withstand autoclaving without loss of activity [2].

Similarly, Panitch *et al.* have incorporated periodically spaced fibronectin CS5 domains into ELPs [60]. These polymers are intended to function as surfaces for vascular grafts and enhanced the attachment of endothelial cells to a glass substrate.

Halstenberg *et al.* have synthesized hybrid protein-graft-poly (ethylene glycol) polymers for tissue repair [61]. The design of this biomaterial included motifs for rationally controlling both cellular attachment and biodegradation. Hydrogels prepared by crosslinking the polymer acted as scaffolds for three-dimensional outgrowth of fibroblasts, which attached to the polymer via specific interactions between cellular integrins and an RGD sequence on the polymer. Outgrowth involved serine protease degradation of the polymer at sites engineered for this purpose.

ELPs have been investigated as materials for cartilaginous tissue repair [62]. Chondrocytes cultured in ELP matrices retained their phenotype and synthesized significant amounts of extracellular matrix molecules that are regarded as important for cartilage function. This work highlights the potential use of genetically engineered polymers as biocompatible extracellular matrices for tissue repair and engineering. It may also be possible to incorporate drugs and/or genes into these systems to guide the development of new tissue.

## **6. Biocompatibility and Biodegradation**

The utility of genetically engineered polymers for biomedical applications will depend, to a large extent, on their biocompatibility and biodegradation. An obvious concern in the use of protein-based materials is the potential activation of an immune response toward the material. In terms of biodegradation, protein-based polymers are thought to degrade to their amino acid constituents, which should be relatively nontoxic and may be used as nutrients. Unfortunately, the biocompatibility and biodegradation of a large number of protein-based materials remains uncharacterized. In the following paragraphs, we highlight some of the characterizations that have been performed to date.

The rate of resorption of polymeric biomaterials can often be adjusted by controlling their composition. Interestingly, it has been shown that the resorption rate of protein-based block copolymers (SELPs) can be controlled by adjusting *both* the *sequence* and *composition* of the copolymer [2]. Control of degradation through the adjustment of polymeric sequence is especially advantageous, because these alterations often do not impact the overall chemical properties of the material and may be made

without significantly impacting its physico-mechanical properties. SELPs have been found to degrade primarily by enzymatic proteolysis (e.g., via elastase) and to produce amino acid degradation products that are largely non-toxic. Little is known about the degradation of the silk-like blocks.

Resorption of subcutaneously implanted SELP films has been evaluated in rats, over the course of seven weeks [2]. A collagen control and SELP-0, with a 4:1 elastin to silk ratio were both resorbed within one week. SELP-8 implants, with a 2:1 ratio of elastin to silk, retained 18% of their initial mass after seven weeks, while SELP-3 implants, with a 1:1 elastin to silk ratio retained 58% of their initial mass. SELP-4 and SELP-5, each containing eight silk-like blocks and 3:2 and 2:1 elastin to silk ratios, respectively, showed no evidence of resorption after seven weeks. These studies demonstrate that the resorption of SELPs is controlled more by the length of the silk-like blocks (i.e., sequence) than the elastin to silk ratio (i.e., composition).

Histological analysis of the implanted films generally showed a mild immune response up to one week, with some macrophage surveillance [2, 39]. Beyond one week, remaining films were observed to be surrounded by a zone of healing tissue, consisting of fibroblasts, collagen, and small numbers of macrophages. SELPs that were resorbed within one week left regions that were diffusely populated by macrophages. Experiments performed with SELP sponges yielded similar results, with no evidence of chronic inflammation or toxicity over 28 days [2].

To investigate their biocompatibility with wounded tissue, SELP-7 and SELP-5 fibrous meshes were applied to porcine dermal wounds. No adverse effects were observed and the wounds (2x2 cm partial and full dermal thickness) were completely

epithelialized after 14 days. Histological evaluation revealed that some SELP filaments had been incorporated into the healing tissue. Similar results were observed with SELP sponges [2].

The immunogenicity of SLP-F and SELP copolymers had been evaluated in rabbits. Compared to hyperimmune positive control rabbit sera, the immunogenicity of all polymers was found to be relatively low. In cases where an elevated antibody titer was observed (e.g., against SLP-F), the antibody response was found to be directed only at the silk-like blocks of the polymer. No response was detected against the elastin-like blocks or fibronectin cellular attachment sites [2].

A broad characterization of the biocompatibility of elastin-like polymers was performed by Urry and colleagues [63]. This study involved applying standard biocompatibility tests to chemically synthesized (VPGVG) with a degree of polymerization greater than 120, and a cross-linked gel formed by gamma irradiation. Results indicated that the two forms of the polymer were non-mutagenic, non-toxic, non-antigenic, non-sensitizing, non-pyrogenic, non-hemolytic, and exhibited favorable compatibility after muscle implantation. These studies indicate that elastin-like polymers exhibit good biocompatibility that is favorable for biomedical applications.

## **7. Conclusions**

The diverse functionality of proteins and the fidelity with which they are biologically synthesized is a resource that is just starting to be investigated by materials scientists. Although the application of genetically engineered polymers for the delivery of bioactive molecules is in its infancy, the level of interest is gaining momentum and it

is likely that new materials and applications will emerge in the near future. A more thorough understanding of protein structure and function will undoubtedly encourage the use of more complicated, stimuli-responsive elements in these materials in drug delivery, gene delivery, and many other biomedically relevant applications.

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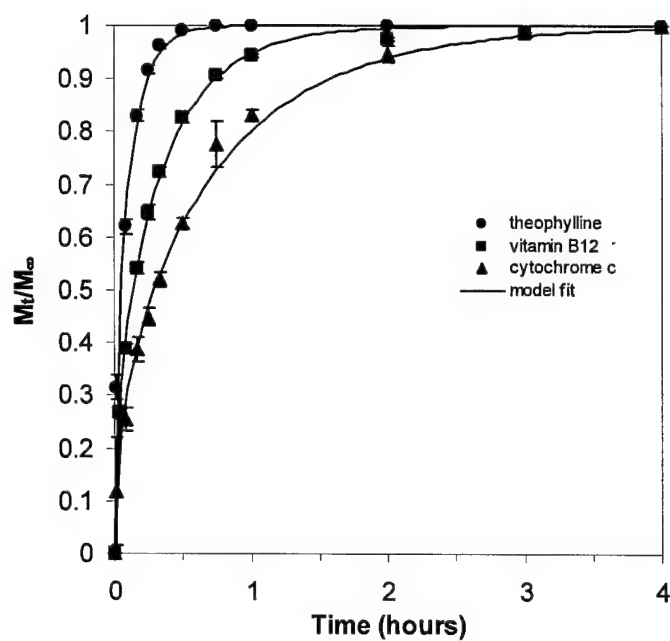
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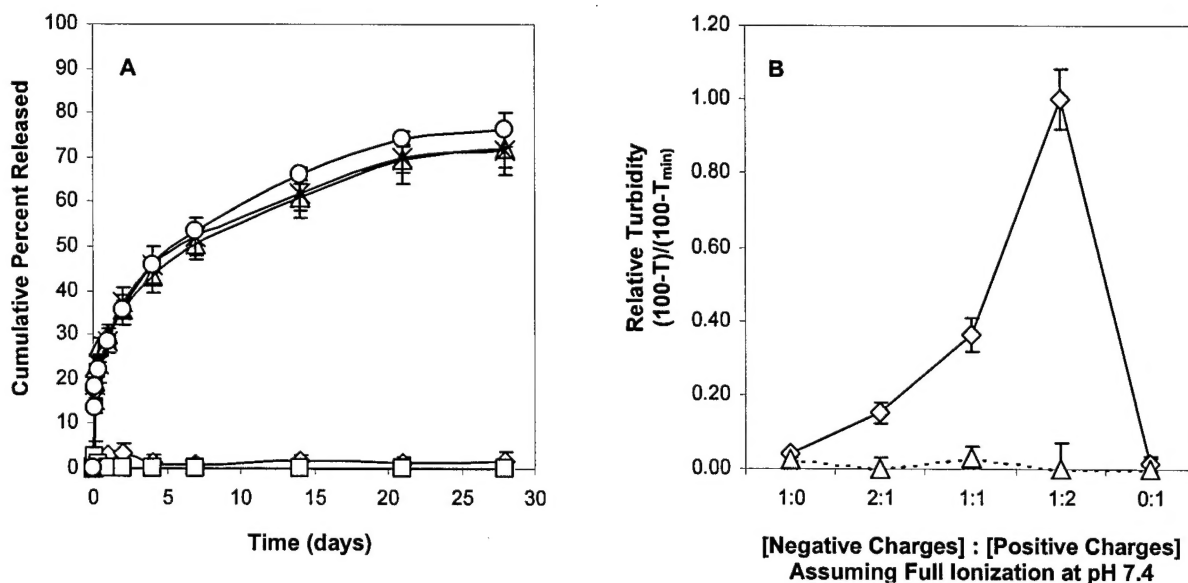
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MDPVVLQRRDWENPGVTQLNRLAAHPPFASDPMGAGSGAGAGS  
[(GVGV**P**)<sub>4</sub>GKG**V**P(GVGV**P**)<sub>3</sub>(GAGAGS)<sub>4</sub>]<sub>12</sub>  
(GVGV**P**)<sub>4</sub>GKG**V**P(GVGV**P**)<sub>3</sub>(GAGAGS)<sub>2</sub>GAGAMDPGRYQDLRSHHHHHH

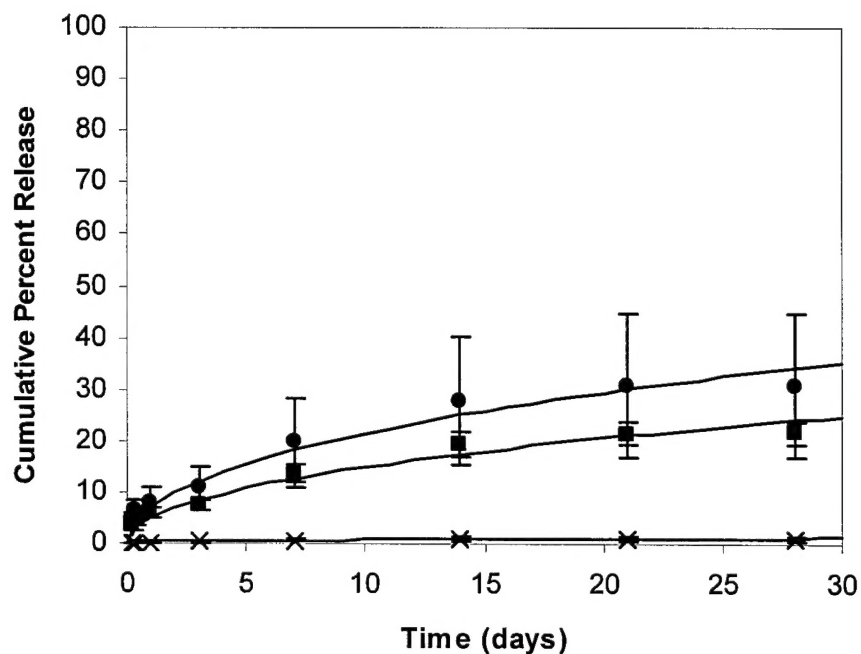
**Figure 1.** The amino acid sequence of SELP-47K. The 884 amino acids have a molecular weight of 69,814 Daltons. It is composed of head and tail portions, and a series of silk-like (GAGAGS) and elastin-like (GVGV**P**) repeats (primary repetitive sequence in bold). Abbreviation key: A=Alanine; D=Aspartic Acid; E=Glutamic Acid; F=Phenylalanine; G=Glycine; H=Histidine; K=Lysine; L=Leucine; M=Methionine; N=Asparagine; R=Arginine; P=Proline; Q=Glutamine; S=Serine; T=Threonine; V=Valine; W=Tryptophan; Y=Tyrosine.



**Figure 2.** Experimental release profiles for solute diffusion from hydrogels prepared from 12 wt% Polymer 47K solutions incubated for 24 hours at 37°C. Symbols represent mean value  $\pm$  one standard deviation ( $n=3$ ). The y-axis denotes the fraction of solute released from the hydrogel at time  $t$ . Reproduced from reference [41] with permission.

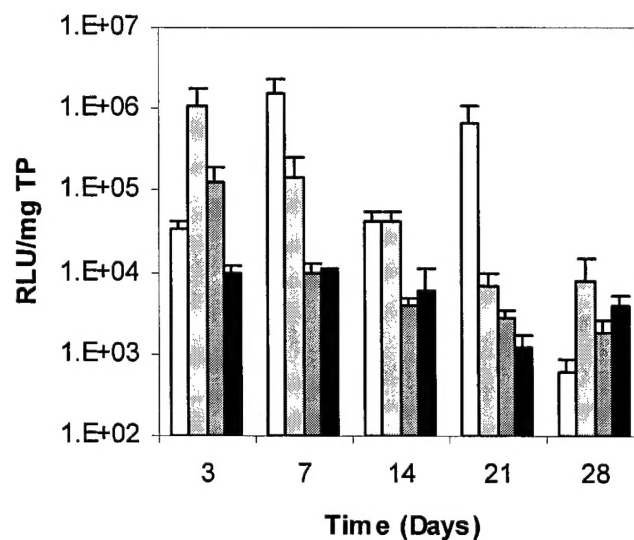


**Figure 3. (A)** Cumulative release of pRL-CMV from 12 wt% SELP-47K hydrogels, in PBS with  $\mu=0.03$  M ( $\diamond$ ),  $0.10$  M ( $\square$ ),  $0.17$  M ( $\triangle$ ),  $0.25$  M (X), and  $0.50$  M (O). Hydrogels were cured for one hour at  $37^\circ\text{C}$  before placement in the appropriate buffer. Each point represents average  $\pm$  standard deviation ( $n=3$ ). **(B)** Effect of ionic strength on the formation of insoluble complexes between SELP-47K and pRL-CMV, in PBS with  $\mu=0.03$  M ( $\diamond$ , solid line) and  $\mu=0.17$  M ( $\square$ , dashed line). Ratios on the x-axis indicate the molar ratio of negative (DNA) charges to positive (polymer) charges, assuming 100% ionization of each at pH 7.4. The y-axis represents relative turbidity  $(100-T)/(100-T_{\min})$ . Data points represent average  $\pm$  standard deviation ( $n=3$ ). Used with permission from reference [40].

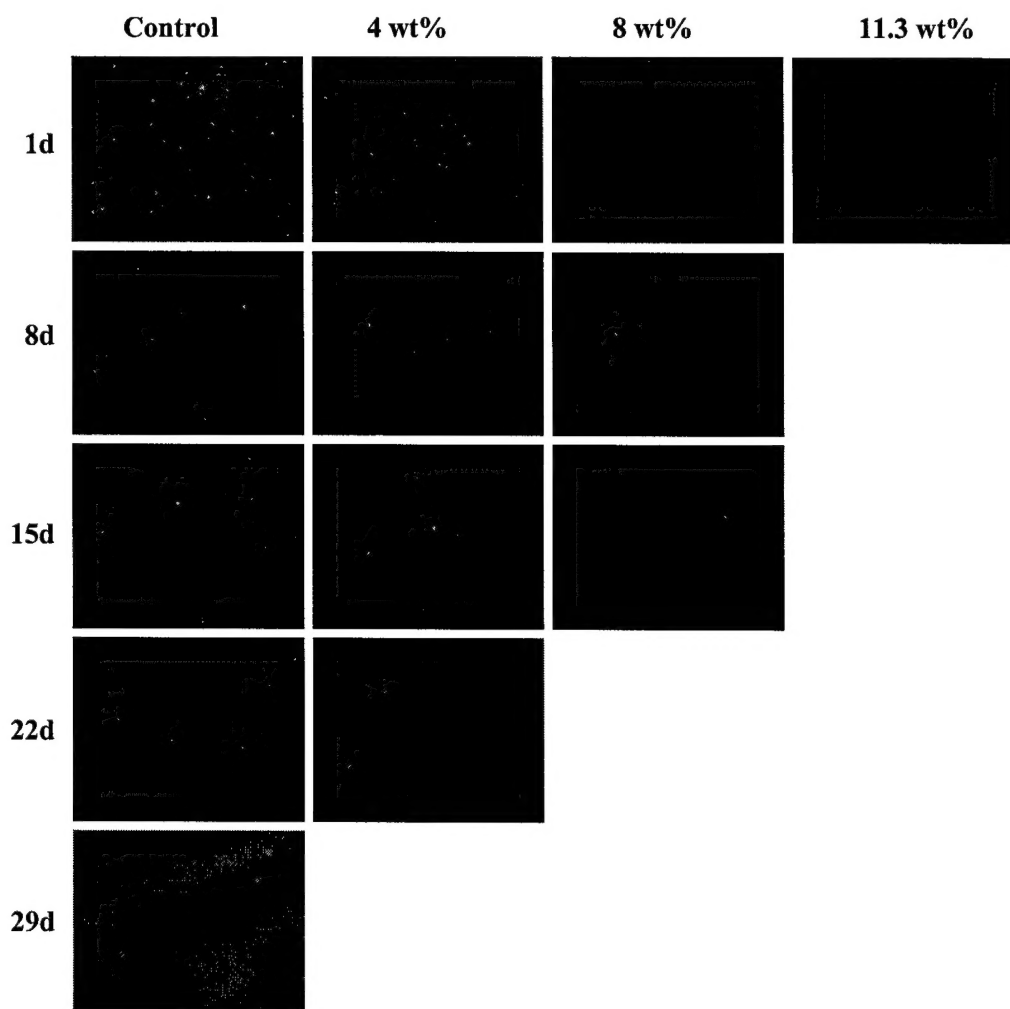


**Figure 4.** Effect of pRL-CMV conformation on *in vitro* release from SELP-47K hydrogels: (●) Linear, (■) Supercoiled, (×) Open-circular; (—) Theoretical release based on model for two-dimensional diffusion from a cylinder (ref. 44). Each data point represents the mean  $\pm$  standard deviation for  $n=3$  samples. Used with permission from reference [50].





**Figure 5.** Expression of *Renilla* luciferase in MDA-MD-435 tumors grown subcutaneously in athymic *nu/nu* mice, after intratumoral injection. Bars represent 4 wt% polymer (white), 8 wt% polymer (light gray), 12 wt% polymer (dark gray), and naked DNA without polymer (black). Each bar represents the mean  $\pm$  standard error of the mean for  $n=4$  or  $n=5$  samples. Used with permission from reference [50].



**Figure 6.** Adenovirus release from SELP-47K and the corresponding bioactivity results. The percentage of polymer increases from left to right. The time of release of virus from gels used to transfect cells or control increases from top to bottom. The images are from fluorescent microscopy at 40X magnification. Bright spots represent individual cells transfected with AdGFP. Used with permission from reference [50].